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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) **World Intellectual Property Organization**
International Bureau

(43) **International Publication Date**
**18 December 2003 (18.12.2003)**

**PCT**

(10) **International Publication Number**
**WO 03/104444 A1**

(51) **International Patent Classification**[7]: C12N 5/08

(21) **International Application Number:** PCT/AU03/00704

(22) **International Filing Date:** 5 June 2003 (05.06.2003)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**

| | | |
|---|---|---|
| PS 2793 | 5 June 2002 (05.06.2002) | AU |
| 2002951874 | 4 October 2002 (04.10.2002) | AU |
| 2003901536 | 28 March 2003 (28.03.2003) | AU |
| 2003901537 | 28 March 2003 (28.03.2003) | AU |
| 2003902348 | 15 May 2003 (15.05.2003) | AU |

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(81) **Designated States** *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 03/104444 A1

(54) **Title:** GENERATION OF NEURAL STEM CELLS FROM UNDIFFERENTIATED HUMAN EMBRYONIC STEM CELLS

(57) **Abstract:** The present invention relates to the generation of neural cells from undifferentiated human embryonic stem cells. In particular it relates to directing the differentiation of human ES cells into neural progenitors and neural cells and the production of functioning neural cells and/or neural cells of a specific type. The invention also includes the use of these cells for the treatment of neurological conditions such as Parkinson's disease.

REC'D 14 AUG 2003

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# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference<br>696039 | FOR FURTHER ACTION | see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. |
|---|---|---|
| International application No.<br>**PCT/AU03/00704** | International filing date *(day/month/year)*<br>5 June 2003 | (Earliest) Priority Date *(day/month/year)*<br>5 June 2002 |
| Applicant<br>ES CELL INTERNATIONAL PTE LTD et al | | |

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 7 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
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2. [X] **Certain claims were found unsearchable** (See Box I).
3. [X] **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   ☐ the text is approved as submitted by the applicant.
   [X] the text has been established by this Authority to read as follows:

   **Generation of neural stem cells from undifferentiated human embryonic stem cells**
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6. The figure of the **drawings** to be published with the abstract is Figure No.
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   ☐ because this figure better characterizes the invention
   [X] None of the figures

# GENERATION OF NEURAL STEM CELLS FROM UNDIFFERENTIATED HUMAN EMBRYONIC STEM CELLS

## FIELD OF THE INVENTION

The present invention relates to the generation of neural cells from undifferentiated human embryonic stem cells. In particular it relates to directing the differentiation of human ES cells into neural progenitors and neural cells and the production of functioning neural cells and/or neural cells of a specific type. The invention also includes the use of these cells for the treatment of neurological conditions such as Parkinson's disease.

## BACKGROUND OF THE INVENTION

Embryonic stem (ES) cell lines are derived from the pluripotent cells of the early embryo. These cell lines, potentially, can maintain a normal karyotype through an infinite life span *in vitro* and their pluripotent stem cells can differentiate into any cell type. ES cell lines derived from human blastocysts allow the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug discovery and screening *in vitro*.

The mammalian nervous system is a derivative of the ectodermal germ layer of the post-implantation embryo. During the process of axis formation, it is thought that inductive signals elaborated by several regions of the embryo (the anterior visceral endoderm and the early gastrula organiser) induce the pluripotent cells of the epiblast to assume an anterior neural fate. The molecular identity of the factors elaborated by these tissues which direct neurogenesis is unknown, but there is strong evidence from lower vertebrates that antagonists of the Wnt and BMP families of signalling molecules may be involved.

Embryonic stem cells are pluripotent cells which are thought to correspond to the epiblast of the pre-implantation embryo. Mouse ES cells are able to give rise to neural tissue *in vitro* either spontaneously or during embryoid body formation. The neural tissue often forms in these circumstances in amongst a mixture of a range of cell types.

However, differentiation to a specific neural cell population is required to realize many of the potential applications of ES cells in regenerative medicine of the central nervous system and neuroscience. Alteration of the conditions of culture, or subsequent selection of neural cells from this mixture, has been used in the mouse system to produce relatively pure populations of neural progenitor cells from differentiating cultures of mouse ES cells. These neural progenitors gave rise to the neuronal and glial lineages *in-vitro*. Transplantation experiments have demonstrated the potential of mouse ES derived neural cells to participate in brain development and to correct various deficits in animal model systems.

Human ES cells have been demonstrated to give rise to neural progenitor cells *in vitro* and have further demonstrated the capability of the progenitors to differentiate *in vitro* into mature neurons. In Reubinoff *et al*, 2000, 2001 PCT/AU99/00990, PCT/AU01/00278 and PCT/AU01/00735 methods are described that allow the derivation of highly enriched (>95%) expandable populations of proliferating neural progenitors from human ES cells. The neural progenitors could be induced to differentiate *in vitro* into astrocyte, oligodendrocyte and mature neurons. Transplantation experiments demonstrated the potential of the neural progenitors to integrate extensively into the developing host mouse brain, to respond to local host cues, and to construct the neuronal and glial lineages *in vivo* (Reubinoff et al., 2001, PCT/AU01/00278).

To derive the neural progenitors, mixed somatic differentiation was induced by prolonged culture of undifferentiated human ES cells without replacement of the mouse embryonic fibroblast feeder layer (Reubinoff et al 2000, 2001 PCT/AU99/00990, PCT/AU01/00278). Under these culture conditions, distinct areas comprised of small piled, tightly packed cells that do not express markers of undifferentiated ES cells or early neuroectodermal progenitors were formed among many other cell types. When these areas were mechanically removed and further cultured in defined media that promote the propagation of neural progenitors, they gave rise to the highly enriched preparations of the neural progenitors.

Recently, others have also reported the derivation of neural progenitors from human ES cells (Zhang et al., 2001, Carpenter et al., 2001). However, these authors induced non-specific mixed differentiation of human ES cells by the formation of embryoid bodies (EBs). Following plating of the EBs and culture in defined medium supplemented with mitogens, enrichment for neural progenitors was accomplished by cell sorting or selective separation following enzymatic digestion. Directed differentiation of human ES cells into neural progenitors and further into specific types of neural cells was not reported by these authors.

Directed differentiation of human ES cells into neural progenitors and further on into specific types of neural cells may be highly valuable for basic and applied studies of CNS development and disease. Controlled differentiation of human ES cells into the neural lineage will allow experimental dissection of the events during early development of the nervous system, and the identification of new genes and polypeptide factors which may have a therapeutic potential such as induction of regenerative processes. Additional pharmaceutical applications may include the creation of new assays for toxicology and drug discovery, such as high-throughput screens for neuroprotective compounds. Controlled generation of neural progenitors and specific types of neurons or glia cells from human ES cells *in vitro* may serve as an unlimited donor source of cells for tissue reconstruction and for the delivery and expression of genes in the nervous system.

Directed differentiation of human ES cells into neural progenitors, has been demonstrated with the bone morphogenetic protein antagonist noggin in Pera et al., 2001 and PCT/AU01/00735. Treatment of undifferentiated human ES cell colonies that were cultured on feeders with noggin blocked differentiation into extra embryonic endoderm and uniformly directed the differentiation into a novel cell type ("noggin cells"). Noggin cells are similar in terms of morphology and lack of expression of markers of undifferentiated stem cells or neural progenitors to the small piled, tightly packed cells that were obtained within a mixture of other cell types in high density cultures.

When noggin cells were transferred to defined culture conditions they gave rise to neural progenitors, neurons and glial cells.

A major application of human ES cells may be their potential to serve as a renewable unlimited donor source of cells for transplantation. However, the potential use of human ES cell derived neural cells in regenerative medicine will depend on their capability to restore function. So far the potential of human ES cell derived neural cells to restore function after transplantation has not been demonstrated.

In the mouse, ES cell derived progeny may be functional. Transplantation of low doses of undifferentiated mouse ES cells into the rat striatum results in their differentiation into dopaminergic neurons and restoration of cerebral function and behaviour in animal model of Parkinson's disease (Bjorklund et al 2002). Nevertheless, it should be noted that teratoma tumors were observed in 5 of 22 transplanted animals and in 6 grafted rats no surviving ES cells were found. Teratoma formation and the relatively low survival rate post transplantation preclude the clinical utilization of this approach.

Parkinson's disease is the second most common neurodegenerative disorder affecting over one million patients in the USA. Pharmacological treatments of the disorder, mainly with L-dopa, have limited long term success and are associated with serious motor side effects. Transplantation of dopaminergic neurons (DA neurons) is an alternative approach that potentially may overcome the drawbacks of pharmacological treatments (Lindvall 1997). Clinical trials of transplantation of fetal derived DA neurons into Parkinson's patients show clinical benefits in some patients (Bjorklund and Lindvall 2000; Freed et al., 2001). Nevertheless, the ethical and practical problems of obtaining sufficient fetal donor tissue severely limit widespread application of this mode of therapy. *In vitro* production of transplantable dopaminergic cells at a large scale could circumvent this drawback. A potential source for the unlimited generation of transplantable dopaminergic neurons *in vitro* is embryonic stem (ES) cell lines.

The potential of ES cells to serve as an unlimited donor source of dopaminergic neurons (DA) has been demonstrated in the mouse ES cell system (Lee et al 2000, Kawasaki et al 2000).

Furthermore, functional recovery following transplantation of mouse ES cell-derived DA neurons into an animal model of Parkinson's disease was recently demonstrated (Kim et al., 2002). However, it is known in the art of biology that murine and human ES cells are different in many aspects. Accordingly, methods that are efficient with mouse ES cells may be unsuitable for human pluripotent stem cells. For example, the cytokine leukemia inhibitory factor (LIF) can support undifferentiated proliferation of mouse ES cells (Robertson E 1987) while it has no effect on human ES cells (Reubinoff et al., 2000).

FGF8 and SHH signals control dopaminergic cell fate in the anterior neural plate. In the mouse, expansion of mouse ES cell derived neural progenitors in the presence of FGF8 and/or SHH significantly increases the generation of DA neurons (Lee et al 2000). The combination of SHH and FGF8 fails to induce significant dopaminergic differentiation of neurons that are derived from human EC cells (NT2/hNT, Stull and Lacovitti 2001). This further enhances the differences between human and mouse. Human EC cells resemble human ES cells (Pera MF 2000), and their lack of response may suggest that pluripotent stem cells from a human origin as opposed to their mouse counterpart do not respond to the SHH/FGF8 combination.

Human ES cells can spontaneously differentiate into tyrosine hydroxylase (TH) producing neurons (PCT/AU01/00278, Reubinoff et al., 2001). However, there has been no demonstrated control for the production of dopaminergic neurons at high yields from human ES cells and more importantly the directed differentiation toward a cell type which has the potential for transplantation and treatment of neurological conditions.

Accordingly, it would be desirable to direct the differentiation of human ES cells toward a useful cell type and to generate the cell type in high yield to improve the chances of successful transplantation.

Therefore, it is an object to overcome some of these practical problems and problems of the prior art.

## SUMMARY OF THE INVENTION

In a first aspect of the present invention there is provided a method of directing the fate of undifferentiated hES cells towards neural progenitor cells *in vitro* said method including the steps of:

culturing undifferentiated human ES cells in a defined serum free medium that contains FGF-2 and an antagonist of bone morphogenic proteins (BMP).

In this method, the differentiation of human ES cells is directed into a neural fate and differentiation towards other lineages is eliminated.

In another aspect of the present invention there is provided a non-committed culture of neural progenitor cells prepared by this method as well as isolated non-committed neural progenitor cell derived from this method.

In yet another aspect of the present invention there is provided a method of directing neural fate in a human embryonic stem (hES) cell *in vitro* said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and

culturing the neural progenitor cell in the presence of a neural fate inducer selected from the group including at least one of Fibroblast Growth Factor (FGF), Sonic Hedgehog Protein (SHH), cAMP inducers, Protein Kinase C (PKC) inducers, dopamine and ascorbic acid (AA) or any combination thereof.

The present method provides for a controlled differentiation of neural progenitors, preferably towards a transplantable neural cell that establishes in a predetermined region of the body. Highly enriched preparations of these cells may be obtained by the methods described herein. The newly derived cells have improved transplantability and are more potent *in vivo*.

This improved potency translates to improved survival and/or function of the differentiated cells upon transplantation.

In another aspect of the present invention there is provided a method of directing neural fate in a human embryonic stem (hES) cell *in vitro* said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and

inducing an overexpression of *Nurr* 1 and/or Lmx1b in the hES cell.

Without being limited by theory, applicants propose that the overexpression of the *Nurr*1 and/or Lmx1b gene can direct the differentiation of hES cells toward a neural fate and DA neurons. Applicants have shown that the hES cells that have differentiated toward the neural fate show an over-expression of the *Nurr*1 gene. The expression is maintained during differentiation into neurons that co-express *Nurr*1 and TH.

The *Nurr* 1 and/or Lmx 1b expression may be induced by any methods available to the skilled addressee. Preferably, the gene(s) are introduced by genetic modification. The gene(s) may be introduced by a suitable vector under the influence of an inducer such that when differentiation is to be effected, expression of the gene may be induced by introduction of the inducer to the cell culture.

In another aspect of the invention, there is provided a method of enhancing the survival of transplanted DA neurons said method comprising

obtaining a neural progenitor cell from a hES cell culture or a cell differentiated from the neural progenitor; and

inducing an expression of GDNF and/or BDNF in the neural progenitor cell or a cell differentiated from the neural progenitor.

Without being limited by theory, Applicants propose that a forced expression of GDNF and/or BDNF by the transplanted hES cells or their neural progeny may enhance the survival of transplanted DA neurones. Preferably the expression is an over-expression above a level that is naturally present.

The neural progenitors may be according to the neural progenitors described above. They may be genetically modified to include vectors that express GDNF and/or BDNF and which may be under the influence of an inducer that can be switched on at an appropriate time to enhance the survival of the transplanted cell.

In yet another aspect of the present invention there is provided a genetically modified hES cell that has been prepared by the methods described above. Preferably, the cell can differentiate to a glial cell and can preferably be directed to differentiate upon forced expression of the Nurr1 and/or Lmx 1b gene and/or the GDNF and/or BDNF survival factors.

The present invention also contemplates transgenic animals having the modified genes.

In a further embodiment, the invention includes methods of treating neural conditions using the genetically modified hES cell, said method comprising transplanting the genetically modified hES cell and inducing the expression of the Nurr1 and/or Lmx 1b gene and/or the GDNF and/or BDNF survival factors.

The method describes "directing neural fate". This term as used herein means to guide the differentiation and development of neural progenitors preferably toward a midbrain fate, or toward neuronal cell types, preferably neurons that show characteristics typical of midbrain neurons. The method may be used to generate any neural progenitor or neuronal subtype including but not limited to hES derived motor neurons, GABAergic, glutmertegic, cholinergic, dopaminergic and serotonergic neurons. The method preferably directs a midbrain neural fate to the neural progenitors derived from hES cells. More preferably the neural progenitor cell is committed to a midbrain fate, tyrosine-hydroxylase (TH) positive ($TH^+$) cell or dopaminergic cell.

In a preferred aspect of the present invention there is provided a method of directing midbrain fate to a hES cell *in vitro*, said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and

culturing the neural progenitor cell in the presence of a midbrain fate inducer selected from the group including any one of FGF-1, FGF-8, FGF-17, SHH, AA, cAMP inducers, PKC inducers and dopamine or any combination thereof.

It is most preferred that the midbrain fate inducer is a combination of FGF-1, FGF-8, FGF-17, SHH, IBMX, forskolin, PMA/TPA and dopamine. Various other combinations may be useful including the combination of:

(i) FGF-8 and SHH, IBMX, forskolin, PMA/TPA and dopamine; or
(ii) FGF-17 and SHH, IBMX, forskolin, PMA/TPA and dopamine; or
(iii) FGF-1, and IBMX, forskolin, PMA/TPA and dopamine; or
(iv) FGF-8 alone; or
(v) FGF-17 alone; or
(vi) IBMX, forskolin, PMA/TPA, and dopamine.

In a further preferred embodiment, the method further includes culturing the neural progenitor in the presence of ascorbic acid (AA) or an analogue thereof.

In an even further preferred embodiment, the method further includes culturing the neural progenitor in the presence of NT4 or equivalent thereof such as NT3.

In yet an even further preferred embodiment, the method includes the further step of:

culturing the neural progenitor cells on poly-D-lysine and laminin.

The neural progenitor may also be in the form of neurospheres.

In another aspect of the present invention there is provided a cell culture comprising neural progenitors committed to a neural fate, preferably a midbrain fate. Preferably the neural progenitors are in aggregates or sphere structures.

More preferably when these aggregates are induced to differentiate at least 30% of them give rise to a significant number (>50) of TH+ neurons. The proportion of clumps containing TH+ cells may increase to at least 60% when the midbrain fate of the progenitors is enhanced by midbrain inducers as detailed above.

Preferably, the neural progenitors with a midbrain fate induced by exposure to midbrain fate inducersexpress mRNA of key genes in the development of midbrain and dopaminergic neurons, and give rise to $TH^+$ neurons or dopaminergic neurons.

In another aspect of the present invention, there is provided an isolated human neural progenitor cell having a committed neural fate more preferably a committed midbrain fate. Preferably the cell can differentiate into a $TH^+$ neuron or a dopaminergic neuron. Most preferably, the cell is prepared by methods described herein and isolated from a culture of differentiated hES cells that have been induced to differentiate toward a midbrain fate by the use of midbrain fate inducers described herein.

In another aspect of the present invention, there is provided an isolated human neuronal cell having a committed neural fate, more preferably to a committed midbrain fate. Preferably the cell is $TH^+$ neuron or a dopaminergic neuron. Most preferably, the cell is prepared by methods described herein and isolated from a culture of differentiated hES cells that have been induced to differentiate toward a midbrain fate by the use of midbrain fate inducers described herein.

In another aspect of the present invention there is provided a human neural fate inducer composition for inducing neural fate in a cultured hES cell, said composition comprising a neural fate inducer selected from the group including Fibroblast Growth Factor (FGF), ascorbic acid (AA), Sonic Hedgehog Protein (SHH), cAMP inducers, Protein Kinase C (PKC) inducers and dopamine or any combination thereof.

In yet another aspect of the present invention, there is provided a method of treating a neurological condition in an animal, said method comprising administering an effective amount of an *in vitro* derived neural progenitor cell to the animal. Preferably, the neural progenitor is derived from an undifferentiated hES cell and is not committed to a neural fate. More preferably the neural progenitor cells have a committed neural fate. Most preferably, the neural progenitors are committed to a midbrain fate.

The commitment of the neural progenitors to a midbrain fate may be determined by demonstrating that the neural progenitors express key genes in the development of midbrain and dopaminergic neurons *in vivo.*

In a preferred embodiment, the neurological condition is Parkinson's disease.

## FIGURES

Figure 1 shows dark field images of hES colonies treated with noggin two weeks after passage. (A) A colony mainly comprised of areas of small tight cells (B) A colony with presumably neural rosettes.

Figure 2 shows characterization of cells within areas that are presumably neural rosettes. Fluorescent images of immunostaining for the markers (A) N-CAM and (B) nestin. A very high proportion of the cells express these markers.

Figure 3 shows phase contrast micrograph of neurospheres at various times after derivation. The spheres gradually acquired in culture a round uniform appearance. Spheres generated from noggin treated colonies at 15 (A) and 27 (B) days after derivation. Spheres at 1 (C) and 7 (D) days after transfer of hES cell clumps to neural progenitor medium supplemented with noggin.

Figure 4 shows characterization of the phenotype of cells within spheres three weeks after transfer of hES cell clumps into neural progenitor medium with or without noggin. The proportion of cells that express markers of neuroectoderm, endoderm and mesoderm was evaluated by immunostaining. A high percentage of the cells within the spheres express early neural markers. Noggin treatment

reduced in a dose dependent manner the percentage of cells expressing markers of endoderm and mesoderm.

Figure 5 shows dark field images of clumps of undifferentiated ES cells, and differentiated clumps after culture (3 weeks) in NPM supplemented with FGF2 and noggin (a), the same medium without noggin (b), and knockout medium (c). Clumps from groups a and b were further cultured 3 weeks in NPM supplemented with FGF2 in the absence of noggin.

Figure 6 shows indirect immunofluorescence analysis of the percentage of cells expressing neural markers within hES cell clumps after three weeks of culture in KO medium, NPM+FGF2 and NPM+FGF2+noggin.

Figure 7 shows indirect immunofluorescence analysis of the percentage of cells expressing neural markers within hES cell clumps that were cultured 3 weeks in NPM+FGF2 or NPM+FGF2+noggin followed by additional 3 weeks in NPM+FGF2.

Figure 8 shows indirect immunofluorescence analysis of the percentage of cells expressing endodermal (probably extraembryonic) and mesodermal markers within hES cell clumps after three weeks of culture in KO medium, NPM+FGF2 and NPM+FGF2+noggin.

Figure 9 shows indirect immunofluorescence analysis of the percentage of cells expressing endodermal (probably extraembryonic) and mesodermal markers within hES cell clumps that were cultured 3 weeks in NPM+FGF2 with or without noggin followed by additional 3 weeks in NPM+FGF2.

Figure 10 shows fluorescent images of differentiated clumps of neural cells after treatment with FGF8, SHH and AA and plating on laminin in the presence of AA. A large proportion of the cells express the neuronal marker β-tubulin type III (A, D red). A significant number of the cells are immunoreactive with anti TH (B, E, green). Images of double staining for both markers show that TH+ cells coexpress β-tubulin type III (C, F, yellow)

Figure 11 shows fluorescent images of differentiated clumps of neural progenitors after treatment with FGF17 and AA. A significant proportion of the cells are immunoreactive with anti TH (A-C) while sparse TH+ cells are observed within non-treated clumps (D, E).

Figure 12 shows the effect of various combinations of external factors on the proportion of TH+ clumps. Clumps with > 50 TH+ cells were scored as TH+ ones. Each bar represents 2-3 experiments. 50-150 clumps were scored in each experiment.

Figure 13 shows the effect of treatment with FGF-1, IBMX, forskolin, PMA (TPA), dopamine and AA on the generation of TH+ clumps. Clumps with > 50 TH+ cells were scored as TH+ ones. Each bar represents the scoring of 65-200 clumps.

Figure 14 shows a confocal microscopy image of double immunostaining for TH (green) and β-tubulin type III (red).

Neurospheres that were propagated for 5 weeks, were plated on laminin and allowed to differentiate for a week in the presence of ascorbic acid. The image is a projection of multiple confocal microscopy images of consecutive planes through the differentiating clumps of neural cells.

Figure 15 shows the percentage of neurons expressing TH following treatment with FGF8 and AA. Neural spheres were plated on laminin in the absence of mitogens and treated with AA, FGF8 or both AA and FGF8 for a week. The neural spheres were then further cultured for an additional week in the presence of AA. Each bar represents confocal imaging analysis of 150-300 cell bodies for the expression of TH and β-tubulin III within 10-15 random fields.

Figure 16 shows indirect immunofluorescence images of differentiated neurons decorated with anti TH and anti DAT antibodies.

Figure 17 shows indirect immunofluorescence images of a differentiated neuron coexpressing Nurr1 and TH (A). The neuron was developed from transduced hES cells over-expressing Nurr1. Schematic presentation of the lentiviral vector that was used to force the expression of Nurr1 is presented in B.

Figure 18 shows indirect immunofluorescent analysis of TH expression in the 6-OH dopamine lesioned rat striatum and in the intact striatum of contralateral side.

Figure 19 shows the percentage of cells expressing neural progenitor markers within spheres prior to transplantation. Progenitor cells were analyzed by indirect immunofluorescence for the expression of markers 12-24h after disaggregating of spheres and plating on an adhesive substrate. 93-94% of the progenitors expressed the early neural markers and 27% expressed the neuronal marker β-tubulin type III. The bars represent results from three independent experiments.

Figure 20 shows RT-PCR analysis of expression of regulatory genes of development of ES cells, early CNS, midbrain and dopaminergic neuron by neural progenitors and their differentiated progeny. The symbols + and – indicate whether the PCR reaction was done with or without the addition of reverse transcriptase. HES- mainly undifferentiated hES cell colonies; NPs-neural progenitors after 6 weeks in culture and prior to transplantation.

Figure 21 shows apomorphine-induced rotational behavior in individual sham operated and human neurosphere transplanted Parkinsonian rats. Data is given as percent change in comparison to each rat rotational behavior at 2 weeks after transplantation. At this time point the rats exhibited the full effect of the 6-hydroxydopamine lesions as determined by apomorphine induced rotational behavior. At 12 weeks, all sham-transplanted rats (Ctrl 1-4) showed no difference in rotational behavior as compared to baseline. In 4 out of 5 human neurosphere transplanted rats (ES 2-5) there was a significant decrease in rotational behavior.

Figure 22 shows apomorphine-induced rotational behaviour in individual sham operated and human neurosphere transplanted Parkinsonian rats. In this experiment, neurospheres that were passaged for 5 weeks prior to transplantation were used. Data is given as percent change in comparison to each rat rotational behaviour at 2 weeks after transplantation. At this time point the rats exhibited the full effect of the 6-hydroxydopamine lesions as determined by apomorphine induced rotational behaviour. At 8 weeks, all sham-transplanted rats (Ctrl 1-3) showed no difference in rotational behaviour as compared to baseline. In all 5 human neurosphere transplanted rats (ES 1-5) there was a significant decrease in rotational behaviour.

Figure 23 shows apomorphine-induced rotational behaviour in sham operated and human neurosphere transplanted Parkinsonian rats. At 2, 4, 8 and 12 weeks after transplantation, the severity of the disease was scored and compared between hES cell- transplanted (♦; n=16) and vehicle- transplanted (□; n=12) animals by quantification of rotational behaviour in reaction to apomorphine.

Data is presented as percent change (mean ±SEM) in comparison to rotational behaviour at 2 weeks after transplantation. At this time point, the rats exhibited the full effect of the 6-OH-DA lesions. A significant decrease in rotational behaviour was observed in transplanted animals (70% of baseline versus 105% in controls, at 12 weeks after transplantation, $p<0.05$, student t-test).

Figure 24 shows rotational behaviour in response to amphetamine. The number of amphetamine-induced rotations was significantly lower in neural sphere- transplanted animals (n=11) compared to sham operated control animals (n=10) ($P<0.004$, student t-test).

Figure 25 shows the results of non-pharmacological behavioural evaluation of hES cell-transplanted rats. In both stepping adjustments and forelimb placing non-pharmacological tests there is a significant increase in mobility after stem cell therapy ($P<0.003$, student t test).

Figure 26 shows fluorescent images of a trail of human cells, identified by a human specific anti-mitochondrial antibody along the transplantation tract in the Parkinsonian rat striatum, at 24 hour post transplantation (A) and 1 month post transplantation (B). Arrows indicate areas of recipient striatum, near the transplantation tract, without anti-mitochondria+ cells.

Figure 27 shows fluorescent images of the striatum and the injection tracts after immuno-staining for the neural progenitor marker nestin. Many of the transplanted human cells are in a progenitor state, as indicated by expression of the intermediate filament protein nestin.

Figure 28 shows anti PCNA immunostaning of cells within the neural progenitor grafts. At 24 hours after transplantation the majority of cells expressed PCNA (red), while sporadic expression was observed after 12 weeks. Nuclei were stained with DAPI (blue).

Figure 29 shows fluorescent images of transplanted human cells expressing TH and DAT. (A-D) Double immuno-fluorescent staining demonstrating TH+ cells (red) that are immunoreactive with the anti-human specific mitochondria antibodies (green) within the graft of human neural cells 3 months after transplantation. (A) Low magnification image demonstrating TH+ cells predominantly in the edges of the trail of engrafted cells. (B) High magnification image of the TH+ cells within the graft (nuclei are counterstained with DAPI ((blue). (C-D) Confocal microscopy images of single cells that are co-expressing human mitochondrial antigen (C) and TH (D, nuclei are indicated by asterisks). Cells immunoreactive with anti- human DAT (green), within the lesioned striatum, are demonstrated in (E) (nuclei are counterstained with DAPI (blue)).

Figure 30 shows RT-PCR analysis of expression of human specific midbrain and dopamine neuron markers within brain samples from the area of the graft. The human specific transcripts were expressed in stem cell transplanted animals (n=2) while they were not detected in a control vehicle transplanted animal.

Figure 31 shows the derivation and characterization of spheres. Dark field stereo-microscope images of an undifferentiated hES cell colony one week after passage (A), noggin treated colony at two weeks after passage (B), and hES cell derived spheres (C). Indirect immunofluorescence staining of the progenitor cells, 12 hours after disaggregating of spheres and plating on adhesive substrate, for PSA-NCAM (D), A2B5 (E), N-CAM (F), and nestin (G), demonstrated that >90% of the progenitors within the spheres expressed markers of neural progenitors (J). Following spontaneous differentiation, 30% of the progenitors differentiated into neurons and were immunoreactive with β-tubulin III (J, H and I(red)). Double immunolabelling showed that 0.5% and 1% of the cells co-expressed β-tubulin III (red) and TH (green, H) or serotonin (green, I).

Figure 32 shows human ES cell-derived NPs express key regulatory genes of midbrain development. Semi-quantitative RT-PCR analysis demonstrated the expression of transcripts of key regulatory genes in midbrain and dopaminergic neurons development as well as markers of dopaminergic neurons within cultures of both undifferentiated and differentiated NPs. Transcripts of OCT4, which is a marker of undifferentiated hES cells were not expressed by the NPs. The symbols + and – indicate whether the PCR reaction was done with or without the addition of reverse transcriptase.

Figure 33 shows immunohistochemical characterization of transplanted hES-derived neural cells in the brain. Transplanted cells were identified by human-specific antibodies. Staining with human specific anti-mitochondria antibody (A, green) showed the linear graft within the striatum. The human origin of the graft was confirmed by staining with a human-specific anti ribonuclear protein antibody (B, red), that was localized to the cell nuclei (insert, counterstain with Dapi in blue). At 24 hr post transplantation there was high expression of nestin in the graft (C). At 12 weeks post transplantation there were graft-derived neurons in the rat brains, as indicated by double stainings for human specific markers and neuronal markers. Low power field of slides double stained with human mitochondria (D, green) and neurofilament (D, red) showed that the majority of transplant did not stain with the neuronal marker. There were some

neurofilament+ human cells (D, insert), especially near the interface with the rat brain tissue. Also, there were human RNP+ cells (E, red) that co-labelled with the neuronal marker NeuN+ (E, green). The generation of dopaminergic neurons by the graft was indicated by the presence of TH+ fibers (F, red) within the human mitochondria+ graft (F, green; insert as high power field). Con-focal microscopy confirmed the presence of human mitochondria+ cells (G) co-staining for TH (H). Generation of dopaminergic neurons was confirmed by staining with antibodies directed against human dopamine transporter (I). Also, there were human mitochondria+ cells (J) that co-labelled with V-MAT (K). At 24 hours post transplantation the majority of transplanted cells expressed the proliferative marker PCNA (L, in red over a blue Dapi counterstain). At 12 weeks almost no PCNA+ cells were found (M, blue dapi counterstain without red PCNA stain). Space bars: A-D, F, 50μm; E, G-M, 10μm.

Figure 34 shows RT-PCR analysis of striata samples from sphere and vehicle-grafted animals for the expression of human-specific transcripts of midbrain and dopaminergic neuron markers. The human-specific transcripts were expressed only by animals (n=3 animals) that received hES-derived NPs and were not detected in animals that received sham operation (n=2 animals). Human-specific primers were used to detect transcripts of *En1, En2,* TH, AADC and GAPDH. The β-actin primers were not human specific. The symbols + and – indicate whether the PCR reaction was done with or without the addition of reverse transcriptase.

Figure 35 shows transplantation of hES cell-derived neural spheres improves motor function in Parkinsonian rats. The number of d-amphetamine- or apomorphine- induced rotations was calculated individually for each rat as percentage of its performance at baseline. For each time point the value represents the mean±SE percent rotations. Rotational behaviour that was induced by d-amphetamine (A) and apomorphine (B) decreased significantly in transplanted animals as compared to baseline and to control rats. *p<0.05 as compared to baseline and to controls for the pharmacological tests and to the control group for the non-pharmacological tests. Stepping (C, p=0.0012) and

placing (D, $p=0.0003$) also improved significantly in transplanted rats as compared to controls.

## DESCRIPTION OF THE INVENTION

In a first aspect of the present invention there is provided a method of directing the fate of human embryonic stem cells towards neural progenitor cells *in vitro* said method including the steps of:

culturing undifferentiated human ES cells in a defined serum free medium that contains FGF-2 and an antagonist of bone morphogenic proteins (BMP).

In this method, the differentiation of human ES cells is directed into a neural progenitor and differentiation towards other lineages is substantially eliminated. These neural progenitors are not committed at this stage and have the potential to become committed if placed under conditions to induce commitment.

The direction of the differentiation is influenced by the use of the defined serum free media in particular by the presence of FGF-2 and the BMP antagonist. The present invention provides a method of inducing neural progenitor cells from undifferentiated hES cells such that the process that directs neural progenitor cells is augmented toward a neural lineage whereas other lineages are eliminated or less prominent.

Preferably the BMP antagonist is selected from the group including a direct antagonist such as fetuin, noggin, chordin, gremlin, follistatin, Cerberus, amnionless, DAN or the ectodomain of BMRIA (a BMP receptor protein), or ligand binding domains from other BMP receptors. Preferably the BMP antagonist is noggin.

As previously described in the applicants own application PCT/AU01/00735, noggin used in combination with hES stem cells produces a neural progenitor culture. However, the culture is a mixture of neural progenitors and other cell types. This aspect of the present invention refines the differentiation to neural

progenitors and neural cell types by the use of defined medium conditions which preferably includes the use of FGF-2 with or without noggin.

In another aspect of the present invention there is provided a cell culture comprising neural progenitor cells differentiated from hES cells and wherein the neural progenitors are non-committed to a neural fate. These cultures have the potential to commit to a neural fate.

In another aspect of the present invention, there is provided an isolated neural progenitor cell differentiated from a hES cell and wherein said neural progenitor is not committed to a neural fate. This cell type has the potential to commit to a neural fate.

The neural progenitor cells prepared by this process may be non-committed neural progenitors that are not committed to any particular type of neural cell such as but not limited to neuronal and glial cell types. These cells may be used, as described below and induced to commit to a neural fate and neuronal cell type preferably including a midbrain cell type. Preferably these cells have a potential to commit to a neural fate.

The characteristics and phenotype of cells following differentiation and propagation in culture may be analysed for the expression of the early neural markers such as, but not limited to nestin, A2B5, N-CAM, PSA-NCAM and β-tubulin III. The analysis may be conducted at a suitable time to monitor the progression of the cells through the differentiation process. The cells may be analysed after 3 to 6 weeks of culture in NPM supplemented with FGF2.

The percentage of cells expressing early neural markers increases over time, preferably over three weeks in culture in NPM+FGF2 in comparison to KO medium. Noggin treatment further significantly increases the percentage of cells expressing the neural markers. Preferably the early neural markers including nestin, PSA-NCAM, A2B5 and NCAM are increased. Preferably at least 75% of the cells in any culture expresses the early neural cell markers after culture with an antagonist of bone morphogenic proteins (BMP). Preferably the antagonist

of bone morphogenic proteins (BMP) is noggin. Preferably, at least 95 to 100% of the cells show an increase in expression of the neural markers.

More preferably, A2B5 expression is increased to at least about 95% of the cells in culture; and NCAM expression is increased to at least about 73% of the cells in culture, more preferably the expression is increased to at least about 90%. After an additional 3 weeks of culture in NPM+FGF2 the percentage of cells expressing most of the neural markers may stablize. The major effect of an additional 3 week culture period may increase the percentage of cells expressing NCAM in the noggin treated clumps.

These cells also show a reduction in the expression of non-neural markers such as but not limited to the endodermal marker alpha-fetal protein, the endodermal marker HNF3α or the epidermal marker keratin-14. Other markers showing reduction in expression include laminin and low molecular weight cytokeratin; muscle actin, smooth muscle actin and desmin

At the RNA level, RT-PCR analysis may be used to confirm that in the noggin treated clumps the expression of the endodermal marker alpha-fetal protein, the endodermal marker HNF3α or the epidermal marker keratin-14 is reduced. Preferably these markers are significantly reduced at approximately 3 weeks and preferably undetectable at 6 weeks.

The cells may also be distinguished by their overexpression of *Nurr-1*. More preferably the cells co-express *Nurr-1* and TH. The expression of *Nurr-1* may be maintained during differentiation into neurons particularly those co-expressing *Nurr-1* and TH.

Preferably, the neural progenitors are obtained from undifferentiated hES cells that are directed to differentiate into neural cells by culture in suspension preferably as clumps in defined serum free culture medium preferably neural progenitor media (NPM) in the absence of feeders. The NPM may be supplemented with FGF-2, with/without EGF and/or LIF.

The NPM may contain DMEM/F12 (1:1), B27 supplementation (1:50), glutamine 2 mM, penicillin 50 u/ml and streptomycin 50 µg/ml (Gibco), and supplemented with 20 ng/ml fibroblast growth factor 2 (FGF2) with or without 20 ng/ml human recombinant epidermal growth factor (EGF), and 10ng/ml human recombinant LIF (R & D Systems, Inc., Minneapolis, MN).

The cells may be cultured in suspension and may be exposed to noggin in the range of 350-700ng/ml.

FGF-2 is used to promote proliferation and prevent the differentiation of the undifferentiated non-committed hES cell derived neural progenitors in the presence of noggin. A suitable FGF-2 concentration is approximately 20ng/ml.

In another aspect of the present invention, there is provided a cultured undifferentiated ES cell which is committed to differentiate to a neural progenitor.

In another aspect of the present invention there is provided a method of directing neural fate in a human embryonic stem (hES) cell *in vitro* said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and

culturing the neural progenitor cell in the presence of a neural fate inducer selected from the group including at least one of Fibroblast Growth Factor (FGF), Sonic Hedgehog Protein (SHH), cAMP inducers, Protein Kinase C (PKC) inducers, dopamine and ascorbic acid (AA) or any combination thereof.

The present method provides for a controlled differentiation of neural progenitors, preferably towards a transplantable neural cell that establishes in a predetermined region of the body. Highly enriched preparations of these cells may be obtained by the methods described herein. The newly derived cells have improved transplantability and are more potent *in vivo*. This improved potency translates to improved survival and/or function of the differentiated cells upon transplantation.

The method describes "directing neural fate". This term as used herein means to guide the differentiation and development of neural progenitors preferably toward a midbrain fate, or toward neuronal cell types, preferably neurons that show characteristics typical of midbrain neurons. The method may be used to generate any neural progenitor or neuronal subtype including but not limited to hES derived GABAergic, glutmertegic, cholinergic, motor neurones, dopaminergic and serotonergic neurons. The method preferably directs a midbrain neural fate to the neural progenitors derived from hES cells.

More preferably the neural cell is a neural progenitor cell committed to a midbrain fate, tyrosine-hydroxylase (TH) positive ($TH^+$) cell or dopaminergic cell.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The neural progenitor cells may be obtained by any means that provides these cells from a culture of hES cells preferably an undifferentiated culture of hES cells. The hES cells may be spontaneously differentiated or induced to differentiate preferably as described in the applicant's own applications namely PCT/AU01/00278 and PCT/AU01/00735, the contents of which are incorporated herein.

Preferably, the neural progenitor cells are non-committed hES cell derived neural progenitors that have not been committed to any particular neural cell type or fate.

In a further preferred embodiment, the neural progenitor cells are obtained herein from a hES cell culture treated with noggin or other inhibitors or antagonists of bone morphogenic proteins (BMP). The use of noggin directs the undifferentiated hES cell within colonies that are cultured on feeders into areas comprised of small tight cells and areas with neural rosettes. Dissection of these areas and transferral into defined serum free culture conditions may

produce characteristic preparations of proliferating neural progenitors. The serum free culture conditions may include media which may be neural progenitor growth medium (NPM) supplemented with fibroblast growth factor (FGF) preferably FGF-2, with/without epidermal growth factor (EGF) and/or leukaemia inhibitory factor (LIF) A typical NPM may contain DMEM/F12 (1:1), B27 supplementation (1:50), glutamine 2 mM, penicillin 50 u/ml and streptomycin 50 μg/ml (Gibco), and supplemented with 20 ng/ml human recombinant epidermal growth factor (EGF), and 20 ng/ml fibroblast growth factor 2 (FGF2) (R & D Systems, Inc., Minneapolis, MN).

In a further preferred embodiment the neural progenitors are obtained from undifferentiated hES cells that are directed to differentiate into neural cells by culture in suspension preferably as clumps in defined serum free culture medium preferably NPM in the absence of feeders. The NPM may be supplemented with FGF-2, with/without EGF and/or LIF.

In an even further preferred embodiment the undifferentiated hES cells that are cultured in suspension as above, are also treated with noggin. Noggin may be used in a range of 350-700ng/ml. The addition of noggin further promotes the differentiation towards the neural lineage and into neural progenitors, while it reduces the differentiation into non-neural lineages. These cultures provide neurospheres comprising neural progenitors that may be used to differentiate toward a neural cell line with a committed neural fate when cultured in the presence of the neural fate inducers.

During the culturing of the hES cells and differentiation towards neural progenitors, these cells may be cultured in the presence of FGF, with/without EGF and/or LIF. Preferably the FGF is FGF-2. A suitable concentration of FGF-2 is approximately 20ng/ml. EGF may be added in the form of naturally produced or recombinantly produced EGF, more preferably human EGF is more suitable for hES cells. A suitable concentration for EGF is approximately 20ng/ml. LIF may be added to promote proliferation of neural progenitors during induction of differentiation when noggin is presented to the cells simultaneously. Under these conditions NPM may be supplemented with FGF-

2 and LIF. LIF is preferably a human LIF and suitably used in a concentration of approximately 10ng/ml.

In another aspect of the invention there is provided a method to block differentiation of hES cells towards non-neural lineages. Exposure of hES cells cultured in NPM to noggin blocks the differentiation to non-neural lineages, preferably mesoderm, endoderm (probably extraembryonic) and epidermal lineages.

Additional culture of the spheres in NPM+FGF2 without noggin further eliminates non neural cells, preferably endodermal (probably extraembryonic) epidermal and mesodermal cells. This is evidenced by the reduction of the expression of non-neural markers.

In another aspect of the present invention there is provided a method of directing neural fate in a human embryonic stem (hES) cell *in vitro* said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and
inducing an overexpression of *Nurr* 1 and/or Lmx1b in the hES cell.

Without being limited by theory, applicants propose that the overexpression of the *Nurr*1 and/or Lmx1b gene can direct the differentiation of hES cells toward a neural fate and DA neurons. Applicants have shown that the hES cells that have differentiated toward the neural fate show an over-expression of the *Nurr*1 gene. The expression is maintained during differentiation into neurons that co-express *Nurr*1 and TH.

The *Nurr* 1 and/or Lmx 1b expression may be induced by any methods available to the skilled addressee. Preferably, the gene(s) are introduced by genetic modification. The gene(s) may be introduced by a suitable vector under the influence of an inducer such that when differentiation is to be effected, expression of the gene may be induced by introduction of the inducer to the cell culture. Preferably the cells are genetically modified using the lentiviral vector transduction system as described in PCT/AU02/0175.

In another aspect of the invention, there is provided a method of enhancing the survival of transplanted DA neurons said method comprising

obtaining a neural progenitor cell from a hES cell culture;

inducing an expression of GDNF and/or BDNF in the neural progenitor cell or a cell differentiated from the neural progenitor.

Without being limited by theory, Applicants propose that a forced expression of GDNF and/or BDNF by the transplanted hES cells or their neural progeny may enhance the survival of transplanted DA neurones. Preferably the expression is an over-expression above a level that is naturally present.

The neural progenitors may be according to the neural progenitors described above. They may be genetically modified to include vectors that express GDNF and/or BDNF and which may be under the influence of an inducer that can be switched on at an appropriate time to enhance the survival of the transplanted cell. The timing may coincide with a period that improves the survival of the cell. Preferably, the GDNF and/or BDNF is induced when the cells are transplanted. However, these factors may be induced during the differentiation stage to enhance their survival.

Differentiated and transplantable hES cells of the present invention may be modified to express Nurr1 and/or Lmx 1b along with GDNF and/or BDNF to provide enhanced survival of transplanted hES cells.

Preferably, the cells differentiate to glial cells. Preferentially, the cells are transplanted as hES cells capable of differentiation and the differentiation is induced *in vivo* in the presence of the induced genes Nurr1 and/or Lmx 1b. Further induction of the survival factors GDNF and/or BDNF may also be present *in vivo.*

In yet another aspect of the present invention there is provided a genetically modified hES cell that has been prepared by the methods described above. Preferably, the cell can differentiate to a glial cell and can preferably be directed

to differentiate upon forced expression of the Nurr1 and/or Lmx 1b gene and/or the GDNF and/or BDNF survival factors.

The present invention also contemplates transgenic animals having the modified genes.

In a further embodiment, the invention includes methods of treating neural conditions using the genetically modified hES cell, said method comprising transplanting the genetically modified hES cell and inducing the expression of the Nurr1 and/or Lmx 1b gene and/or the GDNF and/or BDNF survival factors.

The neural progenitor cells are cultured in the presence of neural fate inducers to induce them to differentiate toward a specific neural progenitor cell preferably committed to a midbrain fate or a neural or neuronal cell type preferably with a committed midbrain fate.

The term “neural fate inducer” is any substance that can direct the neural progenitor toward a neural cell type such as, but not limited to a progenitor of a specific neural fate such as but not limited to midbrain fate, midbrain neurons and any neuronal cell type selected from the group including hES derived GABAergic, glutmertegic, cholinergic, dopaminergic, serotonergic and motor neurons. The substance(s) also promotes survival of neurons such as to promote growth, function, augment activity of functioning cells, enhance synthesis of neurotransmitter substances, enhance activity of naturally occurring nerve growth promoting factors, prevent degeneration of neurons, induce regrowth whilst directing the cell toward a neural fate and enhancing survival of the differentiated neural cell.

The term “FGF” as used herein may include, but is not limited to, FGF-1, FGF-2, FGF-6, FGF-8, FGF-9, FGF-98 and FGF-17, or any biologically active fragment or mutein thereof. Preferably for the induction of human neurons from hES cells, it is preferable to use FGF-1, FGF-8 or FGF-17 alone or in combination. The FGF may derive from any animal, preferably mammalian,

more preferably human. Natural or recombinantly produced FGF, preferably FGF-1, FGF-8 or FGF-17 may be used.

Biologically active variants of FGF are also encompassed by the method of the present invention. Such variants should retain FGF activities, particularly the ability to bind to FGF receptor sites.

FGF activity may be measured using standard FGF bioassays, which are known radioreceptor assays using membranes, a bioassay that measures the ability of the molecule to enhance incorporation of tritiated thymidine, in a dose-dependent manner, into the DNA of cells, and the like. Preferably, the variant has at least the same activity as the native molecule.

The biologically active variants can be FGF analogues or derivatives. The term "analogue" as used herein is an analogue of either FGF or an FGF fragment that includes a native FGF sequence and structure having one or more amino acid substitutions, insertions, or deletions. Analogues having one or more peptoid sequences (peptide mimic sequences) are also included. The term "derivative" as used herein is any suitable modification of FGF, FGF fragments, or their respective analogues, such as glycosylation, phosphorylation, or other additions of foreign moieties, so long as the FGF activity is retained. Methods for making FGF fragments, analogues, and derivatives are available in the art.

In addition to the above described FGFs, the method of the present invention can also employ an active mutein or variant thereof. By the term active mutein, as used in conjunction with an FGF, is meant to include a mutated form of the naturally occurring FGF. FGF muteins or variants will generally have at least 70%, preferably 80%, more preferably 85%, even more preferably 90% to 95% or more, and most preferably 98% or more amino acid sequence identity to the amino acid sequence of the reference FGF molecule. A mutein or variant may, for example, differ by as few as 1 to 10 amino acid residues, such as 6-10, as few as 4, 3, 2 or even 1 amino acid residue providing the FGF activity is maintained.

A new member of the FGF family, FGF 17, was recently discovered (Hoshikawa et al., 1998). Like FGF8 it is predominantly expressed in the developing CNS in the midline region of the forebrain and the midbrain-hindbrain junction. In addition it is expressed in additional distinct expression domains (Xu et al., 1999, Heikinheimo et al., 1994, Crossley et al., 1995). FGF-8 is expressed earlier then FGF-17, whose expression persists a little longer (Xu et al., 2000). While these factors may have a functional relationship in patterning some areas of the brain, the role of FGF-17 in CNS development or its effect on ES cell differentiation is unknown.

"Sonic Hedgehog Protein" (SHH) refers to any sonic hedgehog protein derived from any animal, and functional fragments thereof.

"cAMP Inducers" as used herein, may be selected from any compound that induces cAMP activity either directly by forskolin or NPA (R(-)-propylnorapomorphine a D2 receptor agonist of PKA, increases cAMP) or indirectly by inhibiting phosphodiesterase by Isobutyl-methoylxanthine (IBMX) or by compounds with IBMX like activity such as cAMP-specific Ro 20-1724 , Rolipram , or Etazolate but more preferably selected from the group including Isobutyl-methoylxanthine (IBMX), or forskolin used alone or in combination.

"Protein Kinase C (PKC) Inducers" as used herein may be phorbol myristate acetate or phorobol 12-myristate 13-acetate which is a specific activator of PKC group A ($\alpha$,$\beta$I,$\beta$II,$\chi$) and PKC group B ($\delta$,$\varepsilon$,$\eta$,$\theta$)) or tumor promoter activity (TPA). However, any substance that induces isozyme specific PKC either directly or indirectly is included in the scope of the present invention.

"Dopamine" as used herein includes naturally or synthetically produced dopamine or any functional equivalent or analogue thereof. "Functional equivalents or analogues" are those compounds that have the same activity as naturally produced dopamine that is produced by the adrenal medulla.

Dopamine, along with epinephrine, norepinephrine, and serotonin, belongs to a chemical family referred to "monoamines". Within the family of monoamines,

epinephrine, norepinephrine, and dopamine are derived from the amino acid tyrosine and form a subfamily called the catecholamines. Frequently, tyrosine hydroxylase (TH), the rate-limiting enzyme for the biosynthesis of dopamine, is used as a marker to identify dopaminergic neurons.

In a preferred aspect of the present invention there is provided a method of directing midbrain fate to a hES cell *in vitro*, said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and

culturing the neural progenitor cell in the presence of a midbrain fate inducer selected from the group including any one of FGF-1, FGF-8, FGF-17, SHH, AA, cAMP inducers, PKC inducers and dopamine or any combination thereof.

The neural progenitor may be presented as clumps or clusters of cells, neurospheres or single cells. Clumps or clusters may comprise any numbers of cells preferably providing the cells can be exposed to the neural fate inducers. However, cells on the outer of the clumps may be induced toward a neural fate. Preferably the clumps or neurospheres comprise up to 3000 cells per clump, more preferably the clumps comprise 2000-3000 cells per clump. The neural progenitors may be presented to the inducers either in suspension or adherent cultures, preferably the cells are adherent to poly-D-lysine and laminin.

The use of the specific midbrain fate inducers causes the differentiation to be directed to midbrain cells rather than neural cells of other brain regions.

The neural cells may be cultured in the presence of FGF-2 with or without EGF and/or LIF. When culturing in the presence of the midbrain fate inducers, FGF-2 and EGF and/or LIF may be removed from the medium so that the progenitors are more readily directed to take a midbrain fate by treatment with the midbrain fate inducers.

FGF selected from the group including FGF-1, FGF-8 and FGF-17 is used as a midbrain fate inducer. These factors are preferably used in combination with other midbrain fate inducers at a concentration of approximately 100-200ng/ml.

SHH is preferably used in combination with other midbrain inducers at a concentration of about 0.5-1μg/ml.

cAMP inducers such as IBMX or forskolin may be used at a concentration of about 0.25mM and 50μM respectively in combination with other midbrain inducers.

The PKC inducers PMA/TPA may be used in the concentration of 200nM and dopamine may be used at a concentration of about 20μM in combination with other midbrain inducers.

It is most preferred that the midbrain fate inducer is a combination of FGF-1, FGF-8, FGF-17, SHH, IBMX, forskolin, PMA/TPA and dopamine. Various other combinations may be useful including the combination of:

(i) FGF-8 and SHH, IBMX, forskolin, PMA/TPA and dopamine; or
(ii) FGF-17 and SHH, IBMX, forskolin, PMA/TPA and dopamine; or
(iii) FGF-1, and IBMX, forskolin, PMA/TPA and dopamine; or
(iv) FGF-8 alone; or
(v) FGF-17 alone; or
(vi) IBMX, forskolin, PMA/TPA, and dopamine.

Preferably, FGF-8 and FGF-1 are used in a concentration of about 200ng/ml.

In a further preferred embodiment, the method further includes culturing the neural progenitor in the presence of ascorbic acid (AA) or an analogue thereof.

The addition of AA or an analogue thereof to the cells in the presence of the midbrain fate inducers improves the survival of the cells and further directs the differentiation toward $TH^+$ cells. Furthermore, TH production/expression may be increased. AA or an analogue thereof may be added together with or following

exposure of the cells to midbrain fate inducers. Addition of AA or an analogue thereof at the time of removal of FGF-2 and EGF may improve $TH^+$ generation. The AA or an analogue thereof is supplemented to the medium, preferably NPM in the presence or absence of FGF-2 and EGF and/or LIF. AA or an analogue thereof may be used at a concentration of approximately 400-800μM.

AA or an analogue thereof may be used with any one or combination of FGF-1, FGF-8 or FGF-17 with or without other midbrain inducers.

In an even further preferred embodiment, the method further includes culturing the neural progenitor in the presence of NT4 or equivalent thereof such as NT3.

The term "equivalent thereof" as used herein means a sequence or molecule which functions in a similar way but may have deletions, additions or substitutions that do not substantially change the activity or function of the sequence or molecule.

NT4 is a survival factor like AA. If added at the stage of AA addition, the proportion of $TH^+$ neurons can be increased. NT4 may be used at a concentration of about 20ng/ml.

In yet an even further preferred embodiment, the method includes the further step of:

culturing the neural progenitor cells on poly-D-lysine and laminin.

This additional step will induce further differentiation into neurons. The neural progenitors, having been exposed to midbrain inducers, AA with or without NT4 may be disaggregated at this stage and plated on poly-D-lysine and laminin. Generally, the concentration of poly-D-lysine is in the range of about 5 to 15μg/ml, preferably 10 μg/ml and laminin is in the range of about 1 to 10 μg/ml, preferably, 4μg/ml.

The cells may continue to be cultured in NPM supplemented with AA with or without NT4.

Progression of differentiation throughout the process of the method from undifferentiated ES cells to uncommitted neural progenitors, committed neural progenitors, and specific types of differentiated neural cells may be followed by monitoring the expression of marker genes of various cell types or key genes in the development *in vivo* of undifferentiated ES cells, differentiated cells from various lineages, the CNS, specific areas of the CNS, and various types of differentiated neural cells. The expression of key genes may be monitored at the protein or mRNA level. RT-PCR, semi-quantitative RT-PCR, real time RT-PCR micro and macro arrays or any other method may be used to monitor the expression of mRNA.

Preferably, total RNA is extracted from undifferentiated hES cells; differentiated hES cells; neurospheres at various time points along propagation and following differentiation. RT-PCR is then used to monitor the expression of key genes and markers including: transcriptional markers for undifferentiated hES cells (Oct4); markers of endoderm probably of extra embryonic origin ($\alpha$FP and HNF3$\alpha$); mesoderm marker (CD34); epidermal marker (keratin 14). Early CNS (central nervous system) marker (Otx2); Mesencephalic markers (Pax5, Pax2, wnt1); midbrain markers (*Nurr1, Lmx1b, En1. and En2*) and markers of the dopaminergic pathway (AADC, TH, Ptx3).
Progression of differentiation throughout the process of the method may be monitored also by physical assessment of morphology (ascertained by the trained eye) or by analysis of marker expression at the protein level. Early expression neural markers such as but not limited to N-CAM, A2B5, PSA-NCAM and nestin can help to assess progression toward neural progenitors. . The markers $\beta$-tubulin III, light chain neuro filaments are expressed by early neurons while heavy chain neurofilaments, MAP-2ab, synaptophysin and neurotransmitors are expressed by mature neurons.

Additionally, measurement of $TH^{+}$ may serve as a marker to identify dopaminergic neurons. Other markers of dopaminergic neurons are aromatic – L-amino acid decarboxylase (AADC) and dopamine transporter (DAT). Dopaminergic neurons as opposed to norepinephric neurons lack the

expression of dopamine β hydroxylase (DBH). The production and secretion of dopamine (measured by RP-HPLC) is a definitive marker of dopaminergic neurons. Electrophysiological methods may be further used to characterize the maturity and function of TH+ neurons.

In another aspect of the present invention there is provided a cell culture comprising neural progenitors with a committed fate, preferably a midbrain fate. Preferably the neural progenitors are in aggregates or sphere structures. More preferably when these aggregates are induced to differentiate at least 30% of them give rise to a significant number (>50) of TH+ neurons. The proportion of clumps containing TH+ cells may increase to at least 60% when the midbrain fate of the progenitors is enhanced by midbrain inducers as detailed above.

In a preferred aspect, the cell culture of committed neural progenitors capable of specific neural fate are generated from undifferentiated hES derived neural progenitor cells cultured under defined culture conditions of defined media.

The neural progenitors that are differentiated from undifferentiated hES cells following noggin treatment and specific culture conditions have the potential to give rise to multiple lineages but they also have the potential to further differentiate to cells having a neural fate, preferably a midbrain fate.

The present invention generates cultures of neural progenitors and isolated neural progenitors with a neural fate, preferably a midbrain fate. The neural fate inducers (except the survival factor AA) are removed from the medium at the time of differentiation from progenitors into neurons and still TH+ neurons are obtained. This indicates that the progenitors are committed to a midbrain fate and will give rise to TH+ neurons in the absence of neural fate or midbrain fate inducers. Without being limited by theory, it is considered that the production of progenitors committed to a midbrain fate is important since transplantation of committed progenitors may be more effective than differentiated neurons since they may have a better survival potential after transplantation and may have a higher potential to integrate and interact with the host brain. The committed

progenitors may be identified by their potential to differentiate into TH+ neurons without any treatment with midbrain inducers.

In another preferred aspect of the present invention there is provided a cell culture comprising clumps of differentiated hES cells and wherein at least 30% of the clumps include a significant number (>50) of neurons with a midbrain fate. The proportion of clumps containing TH+ cells may increase to at least 60%.

In another preferred aspect of the invention there is provided a cell culture comprising clumps of differentiated hES cells and wherein at least 30% of the neurons (β-tubulin III+ cells) are expressing TH. The proportion of neurons expressing TH may increase to at least 60%.

In a further preferred aspect there is provided a cell culture comprising a population of differentiated hES cells wherein the population is substantially neural progenitors having a midbrain fate. More preferably, the population comprises neural progenitors that can give rise upon differentiation to neurons that are $TH^+$ or dopaminergic. Most preferably, the cell culture is prepared by the methods described herein.

In an even further preferred aspect there is provided a cell culture comprising a population of differentiated hES cells wherein the population is substantially neurons having a midbrain fate. More preferably, the population comprises neurons that are $TH^+$ or dopaminergic. Most preferably, the cell culture is prepared by the methods described herein.

Preferably, the neural progenitors with a midbrain fate induced by exposure to midbrain fate inducers give rise to $TH^+$ neurons or dopaminergic neurons. Preferably the cells have improved transplant ability and function *in-vivo* wherein improvement is over differentiated hES cells that have differentiated spontaneously into non-committed neural progenitor cells.

Preferably, the cells are functional *in vivo* and more preferably the cells are functional *in vivo* and have the ability to transplant and repopulate by proliferation and differentiation.

By "functional" it is meant to include that the neurons can show nerve growth, be active by enhancing neurotransmitters and by synaptically active and influence motor, sensor cognitive autonomous or any other type of behavior that results from nerve function.

In another aspect of the present invention, there is provided an isolated human neural progenitor cell having a committed neural fate, more preferably a committed midbrain fate. Preferably the cell can differentiate into a $TH^+$ neuron or a dopaminergic neuron. Most preferably, the cell is prepared by methods described herein and isolated from a culture of differentiated hES cells that have been induced to differentiate toward a midbrain fate by the use of midbrain fate inducers described herein.

In another aspect of the present invention, there is provided an isolated human neuronal cell having a committed neural fate, more preferably a committed midbrain fate. Preferably the cell is $TH^+$ neuron or a dopaminergic neuron. Most preferably, the cell is prepared by methods described herein and isolated from a culture of differentiated hES cells that have been induced to differentiate toward a midbrain fate by the use of midbrain fate inducers described herein.

In another aspect of the present invention there is provided a human neural fate inducer for inducing neural fate in a cultured hES cell and blocking non-neural lineages. The inducer is selected from the group of BMP antagonists including but not limited to fetuin, noggin, chordin, gremlin, follistatin, Cerberus, amnionless, DAN or the ectodomain of BMRIA (a BMP receptor protein), or ligand binding domains from other BMP receptors more preferably the inducer is noggin.

In another aspect of the present invention there is provided a human neural fate inducer composition for inducing neural fate in a cultured hES cell, said

composition comprising a neural fate inducer selected from the group including Fibroblast Growth Factor (FGF), ascorbic acid (AA), Sonic Hedgehog Protein (SHH), cAMP inducers, Protein Kinase C (PKC) inducers and dopamine or any combination thereof.

Preferably the composition is a human midbrain neural fate inducer, more preferably the composition is a human midbrain neural progenitor inducer more preferably a $TH^+$ cell inducer, and even more preferably a dopamine producing neuron inducer.

Preferably the FGF is selected from the group including FGF-1, FGF-8 or FGF-17.

Preferably the "cAMP Inducers" are selected from any compound that induces cAMP activity either directly by forskolin or NPA (R(-)-propylnorapomorphine, a D2 receptor agonist of PKA, increases cAMP)or indirectly by inhibiting phosphodiesterase IBMX like activity by cAMP-specific Ro 20-1724 , Rolipram , Etazolate but more preferably selected from the group including Isobutyl-methoylxanthine (IBMX), or forskolin used alone or in combination.

The "Protein Kinase C (PKC) Inducers" may be phorbol myristale acetate (PMA Phorobol 12-myristate 13-acetate is a specific activator of PKC group A $\alpha$,$\beta$I,$\beta$II,$\chi$) and PKC group B ($\delta$,$\varepsilon$,$\eta$,$\theta$)) or tumor promoter activity. However, any substance that induces isozyme specific PKC either directly or indirectly is included in the scope of the present invention.

"Dopamine" may include naturally or synthetically produced dopamine or any functional equivalent or analogue thereof.

In yet another aspect of the present invention, there is provided a method of treating a neurological condition in an animal, said method comprising administering an effective amount of *in vitro* derived neural progenitor cell to the animal.

Preferably, the cells are neural progenitors that have the potential to give rise to multiple lineages and have been derived from undifferentiated hES cell. Preferably, the neural progenitors are committed to a neural fate, more preferably, a midbrain fate. Most preferably, the neural progenitors have been derived from the methods described herein.

The present method can be employed to deliver agents or neural cells to the brain for diagnosis, treatment or prevention of disorders or diseases of the CNS, brain, and/or spinal cord and or peripheral and or autonomic nervous system. These disorders can be neurologic or psychiatric disorders. These disorders or diseases include brain disease such as Alzheimer's disease, Parkinson's disease, Lewy body dementia, multiple sclerosis, epilepsy, cerebellar ataxia, progressive supranuclear palsy, multi-system atrophies, spino-cerebelar degenerations, optic nerve and retinal diseases including retinal and macular degeneration and retinitis pigmentosa, amyotrophic lateral sclerosis, affective disorders, anxiety disorders, obsessive compulsive disorders, personality disorders, attention deficit disorder, attention deficit hyperactivity disorder, Tourette Syndrome, Tay Sachs, Nieman Pick, and other lipid storage and genetic brain diseases and/or schizophrenia. The method can also be employed in subjects suffering from or at risk for nerve damage from cerebrovascular disorders such as stroke in the brain or spinal cord, from CNS infections including meningitis and HIV from tumors of the brain and spinal cord, or from a prion disease. The method can also be employed to deliver agents to counter CNS disorders resulting from ordinary aging (eg anosmia or loss of the general chemical sense), brain injury, or spinal cord injury. The method can also be employed to treat diseases of the peripheral and autonomic nervous systems including but not limited to hereditary neuropathies, inflammatory neuropathies and traumatic neuropathies.

The present method can be employed to deliver agents to the brain for diagnosis, treatment or prevention of neurodegenerative disorders by genetically modifying the hES cells.

The term "treatment" is used in its most broadest sense to include prophylactic (ie preventative) treatment as well as treatments designed to ameliorate the effects of the neurological condition.

An "effective amount" of agent or of hES cells or neural progenitors is an amount sufficient to prevent, treat, reduce and/or ameliorate the symptoms and/or underlying causes of any of the above disorders or diseases. In some instances, an "effective amount" is sufficient to eliminate the symptoms of these disease and, perhaps, overcome the disease itself. When applied to cells, ie effective amount is an amount sufficient to generate cells to prevent, treat, reduce or ameliorate the symptoms.

The *in vitro* hES derived neural cells or neural progenitors are those cells that are either non-committed but are inclined to differentiate toward a neural progenitor cell type or have an induced committed neural fate, preferably neural progenitors more preferably neural progenitors with a committed midbrain fate, or differentiated $TH^+$ or dopaminergic neurons. The cells may be identified by neuronal markers such as DAT, RMP and VMAT2. These markers may be identified in the progenitors or the transplanted cells which have been transplanted. Most preferably, these cells are produced by the methods described herein.

In a preferred embodiment, the neurological condition is Parkinson's disease.

Parkinson's disease (PD) is characterized by the progressive loss in function of dopaminergic neurons. The progressive loss of dopaminergic function interferes with the normal working of the neuronal circuitry necessary for motor control so that patients with PD show characteristic motor disturbances such as akinesia, rigidity and rest tremor. Other symptoms include pain, impaired olfaction, alterations of personality and depression.

According to the invention, neural progenitors or midbrain committed neural progenitors or dopaminergic neuronal cells are generated using the cell culturing methods described above. These cells are then administered to the

brain of the patient in need thereof to produce dopamine and restore behavioural deficits in the patient. Preferably, the cells are administered to the basal ganglia of the patient.

The principal therapeutic target in the brain for Parkinson's is the basal ganglia. Other potential sites are substantia nigra which extends forward over the dorsal surface of the basis peduncle from the rostral border of the pons toward the subthalamic nucleus. In addition therapeutic target areas are also the locus ceruleus which is located in the rostral pons region and the ventral tegmental area which is located dorsomedial to the substantia nigra.

According to the invention, the cells are administered to the patient's or animal's brain. The cells may be implanted within the parenchyma of the brain, as well as in spaces containing cerebrospinal fluids, such as the sub-arachnoid space or ventricles. The cells may be also implanted into sites outside the central nervous system such as but not limited to the peripheral and autonomic nerve and ganglia. "Central nervous system" is meant to include all structures within the dura mater.

Typically, the neural cells are administered by injection into the brain of the patient. Injections can generally be made with a sterlized syringe. The exact size needle will depend on the species being treated, the needle should not be bigger than 1mm diameter in any species. Those of skill in the art are familiar with techniques for administering cells to the brain of a patient.

After the neural cells including but not limited to non-committed neural progenitors, committed neural progenitors, neuronal cells of various types are formed according to the cell culturing method previously described, the cells are suspended in a physiologically compatible carrier. As used herein, the term "physiologically compatible carrier" refers to a carrier that is compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Those of skill in the art are familiar with physiologically compatible carriers. Examples of suitable carriers include cell culture medium (eg, Eagle's minimal

essential media), phosphate buffered saline, and Hank's balanced salt solution +/- glucose (HBSS).

The volume of cell suspension administered to a patient will vary depending on the site of implantation, treatment goal and amount of cells in solution. Typically the amount of cells administered to a patient will be a "therapeutically effective amount". As used herein, a therapeutically effective amount refers to the number of transplanted cells which are required to effect treatment of the particular disorder. For example, where the treatment is for Parkinson's disease, transplantation of a therapeutically effective amount of cells will typically produce a reduction in the amount and/or severity of the symptoms associated with that disorder, eg, rigidity, akinesia and gait disorder.

It is estimated that a severe Parkinson's patient will need at least about 100,000 surviving dopamine cells per grafted side to have a substantial beneficial effect from the transplantation. As cell survival is low in brain tissue transplantional in general (5-10%) an estimated 1-4 million dopaminergic neurons should be transplanted. It is estimated that a lower number of neural progenitors committed to give rise to dopaminergic neurons will be required to produce a similar therapeutic response.

Examples of the procedures used in the present invention will now be more fully described. It should be understood, however, that the following description is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

## EXAMPLES

**Example 1: Directed differentiation of hES cells into highly enriched cultures of neural progenitors.**

A. Induction of differentiation on feeders with noggin coupled with manipulation of culture conditions.

To derive enriched preparations of neural progenitors, differentiation of human ES cells was directed into neural fate by transfer of undifferentiated hES cell clumps onto fresh feeders and culture in a modified hES cell medium with

reduced serum concentration and in the presence of the BMP antagonist noggin. Specifically, human ES cells (HES-1 cell line, Reubinoff et al 2000, PCT/AU99/00990) with a stable normal (46XX) karyotype were cultured on mitomycin C mitotically inactivated mouse embryonic fibroblast feeder layer in gelatine coated tissue culture dishes as previously described (Reubinoff et al., 2000 PCT/AU99/00990 and PCT/AU01/00278). To induce differentiation, at the usual passage, clumps of undifferentiated ES cells were plated on fresh feeders and cultured in the usual hES medium supplemented with 10% serum (instead of 20%) and 500ng/ml of noggin (R&D systems). Noggin (500ng/ml) was further added to the medium every other day throughout a 6-8 day culture period (3-4 administrations). After 6-8 days, noggin was omitted and the cells were further cultured in the modified medium with 10% serum for additional 4-6 days. At this time about 12-14 days, about 70% of the colonies differentiated mostly into areas that were comprised of tightly packed small cells with a uniform grey opaque appearance under dark field stereo microscope (Fig.1a, PCT/AU01/00278, PCT/AU01/00735). Other colonies differentiated into areas with structures that could resemble primitive neural rosettes (Fig.1b).

Clumps of about 150 cells were mechanically isolated by using the razor-sharp edge of a micro glass pipette or a razor blade from the grey opaque areas and replated in serum-free medium supplemented with human recombinant FGF-2 and EGF. Specifically, the clusters of cells were transferred to plastic tissue culture dishes containing neural progenitors growth medium (NPM) that consisted of DMEM/F12 (1:1), B27 supplementation (1:50), glutamine 2 mM, penicillin 50 u/ml and streptomycin 50 μg/ml (Gibco), and supplemented with 20 ng/ml human recombinant epidermal growth factor (EGF), and 20 ng/ml fibroblast growth factor 2 (FGF2) (R & D Systems, Inc., Minneapolis, MN). Under these culture conditions the clumps formed free floating spherical structures within 24 hours and sequential propagation and expansion of the sphere cultures was possible as previously described (Reubinoff et al., 2001, PCT/AU01/00278) for prolonged periods (6 months).

During the initial weeks in culture, the spheres gradually acquired a uniform morphology (Figure 3 A,B). A detailed analysis of marker expression by the

cells within the spheres was conducted at 3-4, 8 and 12 weeks after derivation. The spheres were disaggregated into single cells that were plated, fixed and analysed by fluorescent immunohistochemistry for the expression of the early neural markers N-CAM, A2B5, PSA-NCAM and nestin. A high proportion of the cells expressed these markers (N-CAM 83±4% A2B5 84±4%, nestin 69±6% PSA-NCAM 74±2%) and the level of expression was stable during the 12 weeks period.

The phenotype of cells within the areas with structures that could resemble primitive neural rosettes (Fig.1b) was also analyzed. These areas were mechanically dissected, and disaggregated. The cells were plated on laminin and cultured in NPM for two days. Immunophenotyping at that point revealed that 95-100% of the cells expressed N-CAM (Fig 2A) and nestin (Fig 2B).

B. Induction of differentiation by culture of ES cell aggregates in serum free conditions in the presence of noggin and FGF2.

In an alternative approach, undifferentiated human ES cell clumps of about 200 cells were transferred into NPM and cultured in suspension in the presence of noggin (350 and 700ng/ml) for approximately 3 weeks. To further induce neural differentiation, proliferation and prevent the differentiation of neural progenitors the medium was also supplemented with human recombinant FGF-2 (20ng/ml). LIF(10ng/ml) was also included in the medium in early experiments. The media was replaced twice a week. Under these culture conditions the clumps of hES cells turned into round spheres within 7 days (Fig 3 C,D).

To evaluate the effect of these culture conditions, we have evaluated the phenotype of cells within clumps following three weeks of culture. We have examined two concentrations of noggin (350 and 700ng/ml) in comparison to culture in NPM supplemented with FGF2 in the absence of noggin. The clusters were disaggregated into single cells or small clumps that were plated, cultured for 4 hours on laminin, fixed and analysed by indirect immunofluorescence for the expression of the early neural markers nestin, A2B5 and N-CAM. Markers of endoderm (probably extraembryonic; low molecular weight (LMW) cytokeratin and laminin) and mesoderm (muscle specific actin and desmin) were examined

after a week of differentiation. Immunofluorescence methods and source of antibodies are described below in section (C).

A high proportion ( ~ 75%) of the cells within the spheres expressed early neural markers. The percentage of cells expressing nestin and A2B5 was slightly higher in the noggin (700ng/ml) treated spheres. Noggin treatment had a profound significant effect on the level of expression of endodermal and mesodermal markers. The proportion of cells expressing endodermal and mesodermal markers was significantly reduced after noggin treatment in a dose dependent manner (Figure 4).

To analyse and differentiate between the effect of NPM supplemented with FGF2 and noggin on the differentiation of the hES cell clusters we have characterized and compared the differentiation of these clusters following culture in three different media (Fig. 5): (a) NPM supplemented with b-FGF and noggin (700ng/ml), (b) the same medium without noggin and (c) knockout (KO) medium. After three weeks of culture a significant difference in the morphology of the clusters was observed. Clusters that were cultured in KO medium had the typical morphology of embryoid bodies (EBs, Fig 5c). Clusters that were cultured in NPM and FGF2 were characterized by cystic structures and areas of dense cells resembling neural spheres (Fig 5b). Clusters that were cultured in the presence of noggin had the typical morphology of neural spheres without cystic structures (Fig. 5a).

Clusters that were cultured in NPM with (Fig 5a) or without noggin (Fig. 5b) were further cultured for an additional 3 weeks in NPM supplemented with FGF2 in the absence of noggin (Fig 5).

To characterize and compare the phenotype of cells within the clusters following differentiation and propagation at the various culture conditions (Fig. 5), the clusters were disaggregated after 3 weeks in culture into single cells or small clumps that were plated, cultured for 4 hours on laminin, fixed and analysed for the expression of the early neural markers nestin, A2B5, N-CAM, PSA-NCAM

and β-tubulin III (Fig. 6). The same analysis was done after additional 3 weeks of culture in NPM supplemented with FGF2 (Fig. 7).

The percentage of cells expressing early neural markers was increased after three weeks culture in NPM+FGF2 in comparison to KO medium. Noggin treatment further significantly increased the percentage of cells expressing the neural markers (95.6% A2B5; 73% NCAM; Fig.6). After additional 3 weeks of culture in NPM+FGF2 the percentage of cells expressing most of the neural markers was stable in both study groups. The major effect of the additional 3 week culture period was an increase in the percentage of cells expressing NCAM in the noggin treated clumps (from 73% to 93%).

The percentage of cells within the clumps that expressed non-neural markers at 3 weeks (Fig. 8) and 6 weeks (Fig. 9) of the same floating cultures as above was analysed. Indirect immuno fluorescence analysis of the expression of non-neural markers was performed following disaggregation of the clumps and one week of differentiation on laminin. The analysis showed a significant reduction in the percentage of cells expressing these markers in the noggin treated cultures after 3 weeks (Fig 8). Cells expressing these markers were undetectable or rare after additional 3 weeks of culture in NPM+FGF2 (Fig 9).

At the RNA level, RT-PCR analysis confirmed that in the noggin treated clumps the expression of the endodermal (probably extraembryonic) marker alpha-fetal protein was reduced/undetectable at 3 weeks. This marker was also undetectable at 6 weeks. The expression of the endodermal (probably extraembryonic) marker HNF3α was also reduced/undetectable at 6 weeks of culture. The expression of transcripts of the epidermal marker keratin-14 was significantly reduced at 3 weeks and undetectable at 6 weeks.

Collectively these data suggest that highly enriched cultures of neural progenitors are obtained when undifferentiated clumps of hES cells are cultured in NPM+FGF2 supplemented with noggin. The percentage of neural progenitors was higher after differentiation in NPM+FGF2 compared to KO medium. This is in line with our previous report (PCT/AU01/00278). Noggin treatment further

significantly increased the process of neuralization and blocked the differentiation to extraembryonic endoderm (as previously described in PCT/AU01/00735) and epidermis. Either noggin or the culture conditions (NPM) or both reduced the differentiation and/or did not promote the survival of mesodermal cells.

C. Immunohistochemistry Studies

In general, for the immunophenotyping of disaggregated neural progenitor cells and differentiated neurons, fixation with 4% paraformaldehyde for 20 minutes at room temperature was used unless otherwise specified. It was followed by blocking with 5% heat inactivated goat serum (Dako) and permeabilization with 0.2% Triton X (Sigma) in PBS with 0.1% BSA for 30 minutes. Samples were incubated with the primary antibodies at room temperature for one hour. Cells were washed three times with PBS with 0.1% BSA, incubated with the secondary antibodies for 30-45 minutes, counterstained and mounted with Vectashield mounting solution with DAPI (Vector Laboratories, Burlingame, CA). Primary antibodies localisation was performed by using mouse anti rabbit IgG and goat anti mouse IgG conjugated to Cy3 or FITC from Jackson Lab. West Grove, (PA: 1:100-1:500), or anti rabbit FITC and goat anti mouse FITC from Dako (1:20-50). Proper controls for primary and secondary antibodies revealed neither non-specific staining nor antibody cross reactivity.

To characterize the immunophenotype of cells within the aggregates, spheres were mechanically disaggregated into single cells or small clumps and plated on poly-D-lysine and laminin in NPM.The cells were fixed after 1 (for analysis of the expression of neural markers) or - 3-7days (for non-neural markers) and examined for the expression of the following markers: laminin (Sigma mouse monoclonal 1:500) and low molecular weight cytokeratin ((cytokeratin 8, Beckton Dickinson, San Jose, CA ready to use) as markers of endoderm; muscle actin (Reubinoff et al., 2000), smooth muscle actin (Dako mouse IgG 1:50) and desmin (Dako mouse clone D33 1:50) for mesoderm; nestin (rabbit antiserum a kind gift of Dr. Ron McKay; 1:25 or from Chemicon rabbit anti human 1:100-200), N-CAM (Dako, Carpinteria, CA; mouse IgG 1:10-20), A2B5 (ATCC, Manassas, VA; mouse clone105 1:10-20) and PSA-N-CAM

(Developmental Studies Hybridoma Bank, mouse undiluted) as markers of neural progenitors; β-tubulin III (Sigma; mouse IgG 1:2000) for early neurons.

Two hundred cells were scored within random fields (at X200 and X400) for the expression of each of these markers and the experiments were repeated at least 3-5 times.

D. Marker characterization by RT-PCR

RT-PCR was performed as previously described (Reubinoff et al., 2001). Primer sequences (forward and reverse) and the length of amplified products were as previously described (Reubinoff et al 2001) for alpha-feto-protein and HNF3α and as follows for keratin 8: ATGATTGGCAGCGTGGAG, GTCCAGCTGTGAAGTGCTTG (390bp).

**Example 2: Directed differentiation of hES cell-derived neural progenitors towards a midbrain fate.**

Neurospheres that were generated from noggin treated ES cell colonies as described above (Example 1) were propagated 21-35 days in NPM supplemented with FGF-2 and EGF. At this point the spheres were chopped into small clumps (2000-3000 cells per clump). FGF-2 and EGF were removed from the medium and the progenitors were directed to take a midbrain fate by treatment with various factors for 6-8 days. During treatment with the various factors the clumps were either cultured in suspension or plated on poly-D-lysine and laminin. The factors included: FGF1, FGF8, FGF17(R&D Systems) at concentrations of 100-200ng/ml SHH (R&D Systems) at 0.5-1 μg/ml. Other signal transduction inducers: cAMP inducers: IBMX (Sigma, 0.25mM), forskolin (Sigma 50μM), PKC inducers PMA (Sigma 200nM) and dopamine (Sigma 20μM) were also used. The medium was also supplemented with the survival factors Ascorbic acid (AA) (Sigma) at concentration of 400-800μM and NT4 (R&D Systems) at concentration of 20ng/ml. The medium was changed every other day. To induce further differentiation into neurons, the spheres were again disaggregated into small clumps and plated on poly-D-lysine (30-70 kDa, 10 μg/ml, Sigma) and laminin (4 μg/ml, Sigma) and cultured in NPM in the absence of the factors and in the presence of AA with or without NT4 for 5-10 days.

Differentiated cells were analysed by indirect immunofluorescence (as described in example 1) for the expression of β-tubulin type III (Sigma, 1:1000-3000) Tyrosine hydroxylase(TH) (Sigma anti mouse monoclonal, 1:250-500 and Pel-Freez anti rabbit polyclonal, 1:50-100) dopamine transporter (DAT;_Chemicon; rabbit polyclobal 1:50-100) and Nurr1 (Santa Cruz, CA; rabbit poly clonal 1::100-200). The proportion of clumps of cells that were comprised of a significant number of TH+ cells (>50 cells) was scored. Double immunostaining for TH and β-tubulin type III was used to analyse the percentage of cells within clumps expressing TH from the total number of neurons (β-tubulin type III + cells). Multiple confocal microscopy images of consecutive planes through the clump were projected into one image for this analysis (Fig. 14) Ten – fifteen random fields were analysed.

The proportion of cells that differentiated into TH+ neurons when the medium was not supplemented with midbrain fate inducers or survival factors was very poor (about 1%, PCT/AU01/00278, Figure 11D, E) and TH+ clumps were not generated. The differentiation into TH+ neurons was poorer with high passage spheres as opposed to low passage ones and therefore low passage (30-35 days in culture) spheres were used in the *in vitro* studies.

Supplementation of the culture medium with ascorbic acid at the time of removal of FGF-2 and EGF as well as during differentiation of the progenitors on substrate gave rise to a significant number of TH+ neurons in 35% of the clumps (Fig. 12 and 13 bar 6) At the cellular level, 45% of the neurons in these cultures expressed TH (Fig. 14, 15).

Treatment with FGF-8 (200ng/ml) in combination with AA significantly increased the proportion of TH+ clumps to a level of 67%. (Fig. 10, 12). Analysis at the cellular level, using confocal microscopy, also showed an increase (to 77%) in the percentage of TH+ neurons from the total number of neurons (Fig. 15). In contrast SHH treatment had no effect on the proportion of TH+ clumps. The effect of SHH was evaluated in combination with AA or in combination with FGF-8 and AA and in both cases it did not increase the proportion of TH+ neurons (Fig 12).

To demonstrate that the TH+ neuron enriched cultures that are generated following treatment with FGF8 and AA include dopaminergic neurons we have examined the expression of DAT. In vertebrates, DAT is exclusively expressed in DA neurons (Lee et al., 2000). Indirect immunofluorescence studies demonstrated cells expressing DAT within the cultures of differentiated TH+ cells (Fig 16). This data suggested that the treatment with FG8 and AA induced differentiation into dopaminergic neurons.

FGF-17 was also found to have the potential to induce differentiation towards a midbrain fate. Following treatment with the combination of FGF-17 and AA 60% of the clumps were comprised of a significant number of TH+ neurons (Fig 12). The inductive effect of FGF-17 was not significantly different from the effect of FGF-8. Fluorescent images of clumps of differentiated neurons following treatment with FGF-17 and AA are demonstrated in Figure 11. A significant proportion of the cells within these clumps express TH.

Directing the differentiation of hES cell derived neural progenitors towards TH+ neurons was also accomplished by using the combination of FGF-1, cAMP activators IBMX and Forskolin, Protein kinase C (PKC) activator PMA, dopamine and AA. Following treatment with this set of factors (FGF-1 at 200ng/ml) 65-75% of the clumps of differentiated neurons contained a significant number of TH+ neurons (Fig 13). The set of these factors was as efficient as FGF-8 and AA or FGF-17 and AA. It should be noted that a 55% proportion of TH+ clumps was obtained when the signal transduction activators (IBMX, Forskolin, PMA) dopamine and AA were used without FGF-1. In contrast to the effect of FGF-1 at 200ng/ml, the addition of FGF-1 or FGF-8 at a concentration of 100ng/ml to the set of these factors did not increase the proportion of TH+ clumps (Fig 13). In these experiments the surviving factor NT4 was added to AA at the stage of final differentiation when the neurospheres were plated on laminin and cultured in NPM.

The transcriptional factor Nurr1 is required for the induction of midbrain DA neurons, which fail to develop in Nurr1-null mutant mice (Zetterstrom et al.

1997). Forced expression of the *Nurr1* gene may be used to direct the differentiation of human ES cell-derived neural progenitors into DA neurons. We have developed a lentiviral vector transduction system for the introduction of stable genetic modifications into human ES cells (Gropp et al., 2003, patent application No. PCT/AU02/0175).

We have used this system to generate hES cells expressing Nurr1. The vector that we have used (pSIN18.cPPT.hEF-1α.Nurr1.hPGK.Puro.WPRE) include the mouse *Nurr1* gene under the control of hEF1 α promoter followed by a selection marker gene (puromycine resistance element) under the control of hPGK promoter. Neural spheres were developed from the genetically modified hES cells, propagated and induced to differentiate in the presence of AA as detailed above. Indirect immunofluorescence analysis demonstrated neurons coexpressing Nurr1 and TH (Fig. 17). It should be noted that it was not possible to demonstrate by immunostaining the expression of Nurr1 by wild type hES cells following induction of differentiation according to the same protocol.

**Example 3: Improvement of behavioural deficit in an animal model of Parkinson's disease following transplantation of hES cell derived neural progenitors.**

A Parkinson's disease model in rats was induced by stereotaxic injection of the neurotoxin 6-hydroxydopamine to cause unilateral nigrostriatal lesions. 8μg/rat of 6-OH dopamine were injected in 4μl into the right Substantia Nigra. Coordinate of injection were P=4.8, L=1.7, H=-8.6.

Two weeks after the injection of neurotoxin the disease severity was examined in each rat individually by administration of apomorphine (25μg/100g body weight) and quantification of contralateral rotational behaviour by computerized rotameter system (San-Diego Instruments). It should be noted that in early experiments, rotations were counted by an observer 4 times every 12 minutes, for three minutes each time after apomorphine administration. Animals with strong baseline rotational behavior (>500 rotations/hour) were selected for transplantation. In these animals immunofluorescent studies of brain sections demonstrated that the injection of the neurotoxin resulted in complete loss of

tyrosine-hydroxylase stained neurons in the ipsilateral striatum, as compared to preserved tyrosine-hydroxylase in the contralateral side (Figure 18).

Human ES derived neural progenitors (as described in Example 1a) were used for transplantation. The phenotype of cells within the neural spheres was characterized prior to transplantation by immunocytochemical studies (as described in Example 1c) and RT-PCR.

For RT-PCR studies, total RNA was extracted from: (1) human ES cell colonies (one week after passage), (2) free-floating spheres after 6 weeks in culture, (3) differentiated cells growing from the spheres at 1 week after plating on laminin in the presence of AA 400 μM (Sigma) and the survival factors NT3 10ng/ml, NT4 20ng/ml and BDNF 10ng/ml (all human recombinants from R&D). Total RNA was isolated using RNA STAT-60 solution (TEL-TEST, Inc., Friendswood TX) or TRI-reagent (Sigma) followed by treatment with RNase-free DNase (Ambion, The RNA company, Austin Texas). The cDNA synthesis was carried out using Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo (dT) as a primer, according to the manufacturers' instructions (Promega, Madison WI). To analyze relative expression of different mRNA, the amount of cDNA was normalized based on the signal from GAPDH mRNA. Levels of marker mRNAs expressed by neural spheres and differentiated cells were compared to that in the undifferentiated hES cells. PCR was carried out using standard protocols with Taq DNA Polymerase (Gibco invitrogen corporation). Amplification conditions were as follows: denaturation at 94°C for 15 seconds, annealing at 55-60 for 30 seconds, and extension at 72°C for 45 seconds. The number of cycles varied between 18 and 40, depending on the particular mRNA abundance. Primer sequences (forward and reverse 5'-3') and the length of amplified products were as follows:

Oct4 -CGTTCTCTTTGGAAAGGTGTTC, ACACTCGGACCACGTCTTTC, 320bp;

Otx2 -CGCCTTACGCAGTCAATGGG, CGGGAAGCTGGTGATGCATAG, 641bp;

Pax2 -TTTGTGAACGGCCGGCCCCTA, CATTGTCACAGATGCCCTCGG, 300bp;

Pax5 -CCGAGCAGACCACAGAGTATTCA, CAGTGACGGTCATAGGCAGTGG, 403bp;
Lmx1B -TCCTGATGCGAGTCAACGAGTC, CTGCCAGTGTCTCTCGGACCTT, 561bp;
Nurr1 -GCACTTCGGCAGAGTTGAATGA, GGTGGCTGTGTTGCTGGTAGTT, 491bp;
En1 -CTGGGTGTACTGCACACGTTAT, TACTCGCTCTCGTCTTTGTCCT, 357bp;
En2 -GTGGGTCTACTGTACGCGCT, CCTACTCGCTGTCCGACTTG, 368bp;
AADC -CTCGGACCAAAGTGATCCAT, GGGTGGCAACCATAAAGAAA, 252bp;
TH -GTCCCCTGGTTCCCAAGAAAAGT, TCCAGCTGGGGGATATTGTCTTC, 331bp;
β-actin -CGCACCACTGGCATTGTCAT, TTCTCCTTGATGTCACGCAC, 200bp;
GAPDH -AGCCACATCGCTCAGACACC, GTACTCAGCGCCAGCATCG 301bp;
Ptx3 (TGGGAGTCTGCCTGTTGCAG,CAGCGAACCGTCCTCTGGG 372bp)

The hES cell-derived neural spheres were transplanted after partial mechanical dissociation of the spheres into small clumps into the striatum of the rats (400,000 cells / animal) with a hamilton syringe along 2 tracts per striatum, using a stereotaxic device. Coordinate for transplantation were A-P=0, L=3.5, H= -7.5 to -4.5 and A=1, L=2, H= -7.5 to -4. Neural spheres that were passaged for 6 weeks (with high potential of generating neurons and specifically dopaminergic neurons) and neural spheres that were passaged for 11 weeks (with a lower potential for generating dopaminergic cells) were transplanted. Control rats underwent sham operation and were injected with saline. Animals received Cyclosporin A treatment (10mg/Kg) throughout the experiment.

At 2 weeks, 1 month, 2 months and 3 months after transplantation, the severity of the disease was scored by pharmacological and non-pharmacological tests and compared between hES cell transplanted and vehicle transplanted animals. Rotations were counted by a computerized rotameter system for 1 hour after S.C. injection of apomorphine (25μg/100g body weight) and for 1 hour after I.P.

d-amphetamine (4mg/kg performed 2 days later) (in early experiments, rotations were counted by an observer as described above). Non-pharmacological tests included the stepping adjustments (Olsson et al., 1995) and forelimb placing (Lindner et al., 1997) tests. The number of stepping adjustments was counted for each forelimb during slow-sideway movements in forehand and backhand directions over a standard flat surface. The stepping adjustments test was repeated three times for each forelimb during three consecutive days. The forelimb placing test assesses the rats' ability to make directed forelimb movements in response to a sensory stimuli. Rats were held with their limbs hanging unsupported. They were then raised to the side of a table so that their whiskers made contact with the top surface while the length of their body paralleled the edge of the tabletop. Normally, rats place their forelimb on the tabletop almost every time. Each test included ten trials of placing of each forelimb and was repeated in three consecutive days. The results of both tests are expressed as percentage of forelimb stepping adjustments and placing in the lesioned side compared to the non-lesioned side. The mean number of rotations and the mean results (in percentage) of non-pharmacological tests were compared between the experimental groups using student t-test.

The rats were then sacrificed and their brains processed for immunohistochemical studies as previously described (Reubinoff et al., 2001) to determine the fate of the engrafted cells. Transplanted human cells were identified by immunohistochemistry for human specific markers, such as anti-human mitochondrial antibody and anti-human ribonucleic protein (Reubinoff et al 2001). Tyrosine-hydroxylase (TH) stains were performed on thick (20-40 micron) sections to quantify TH density in the striatum and substantia nigra as well as on standard 8 micron section to double stain and co-localize with the human specific markers.

A Rabbit anti TH antibody from Chemicon was used (at 1:100) followed by goat anti-rabbit IgG secondary antibody, conjugated to Cy3 (Jackson immunoresearch laboratory PA; 1:500). TH density was quantified using a computerized image analysis system. Graft survival was analyzed by the computerized image analysis system for staining of the human specific markers.

Cell proliferation within the graft was analysed by immunohistochemical evaluation of the percentage of cells that were decorated with anti PCNA (Chemicon, mouse monoclonal; 1:100) or anti Ki 67 (Novocastra Laboratories Ltd UK; rabbit polyclonal 1:100). Differentiation of transplanted cells into dopaminergic neurons was confirmed by immunohistochemical studies using anti human DAT (Chemicon; rat monoclonal 1:2000) followed by FITC conjugated goat anti rat (Molecular Probes)

Prior to implantation into Parkinsonian rats, the phenotype of the cells within spheres that were propagated 6 weeks in culture was characterized as well as their developmental potential to give rise to midbrain DA neurons. Indirect immunofluorescence analysis following disaggregation of the spheres demonstrated that >90% of the cells within the spheres expressed markers of neural progenitors (Figure 19). Thus, as demonstrated above, the sphere cultures were highly enriched for neural progenitors.

Successful differentiation of the hES cell derived NPs into midbrain DA neurons probably require the induction of the same key regulatory genes that are expressed by neural progenitor cells during the development of the midbrain *in vivo* (Lee et al., 2000). Among these key genes are the *OTX* homebox genes (*OTX1* and *OTX2*) that are widely expressed at the early stages of neuroectoderm differentiation. Interactions between the *OTX* genes are thought to specify the development of the midbrain and hindbrain (Simeone 1998; Acampora D and Simeone 1999). The genes *Pax2, Pax5, Wnt1, En1* and *En2* that are expressed further downstream during midbrain development and serve as early organizers surrounding the ventral midbrain progenitors neurons (Stoykova, & Gruss 1994; Rowitch & McMahon 1995). Lastly, the transcription factors *Nurr1 and Lmx1b* that are implicated in the final specification of the mesencephalic dopamine systems (Zetterstrom et al., 1997; Smidt et al., 2000). These regulatory genes were all expressed by the progenitors within the spheres. The level of expression of these regulatory genes was up regulated upon differentiation (Fig 20). These findings suggested that the neural progenitors had the developmental potential to give rise to midbrain DA neurons. Some of the genes were also weakly expressed by cells within the

hES cell cultures probably reflecting early background neural differentiation. It should be noted that Oct4 was not expressed by the sphere cultures suggesting that the spheres did not include undifferentiated hES cells.

In early experiments we have compared the rotational behaviour of a limited number of rats following transplantation of early passage and late passage neural spheres.

In the experimental group that was transplanted with human neural spheres that were passaged for 11 weeks prior to transplantation there was a mild but statistically significant clinical effect at 3 months post-transplantation (Figure 21). As compared to baseline apomorphine-induced rotational behavior, the control animals exhibited 100.25±9% rotation, while the transplanted animals developed 79±19% rotations ($p=0.04$).

In the experimental group that was transplanted with human neural spheres that were passaged for 6 weeks prior to transplantation, a clinical effect was observed as early as 1 month after transplantation. At this time point the rotational behavior in transplanted animals had already decreased to 72±14% of baseline (as compared to 102±18% in the control group, $p=0.04$). Moreover, the effect of apomorphine in the transplanted animals lasted a shorter length of time, with rotational behavior at 30 minutes after injection of apomorphine reducing to 65±12% of control ($p=0.01$). At 2 months after transplantation the rotational behavior in transplanted rats decreased further to 58% of baseline (versus 102% in controls, $p=0.006$, student t-test). Again, the shortening of length of time of rotational behavior was evident, as after 40 minutes, the transplanted animals exhibited 43% rotations as compared to control ($p=0.005$, Figure 22).

These results are in line with in-vitro studies and suggest that human neural spheres (of early passage) that have a higher potential to generate dopaminregic cells in-vitro also induce a stronger and more rapid clinical improvement in the Parkinsonian rats.

We have therefore transplanted in following experiments only early passage (6 weeks) spheres.

To further study the behaviour of Parkinsonian rats after stem cell transplantation we have extended the number of transplanted and control animals, included analysis of rotation after administration of both apomorphine and amphetamine and evaluated the behaviour of animals in non-pharmacological tests. It should be noted that non-pharmacological tests provide a more direct measure of motor deficits analogous to those found in human Parkinson's disease (Kim et al., 2002). The results of the pharmacological tests are presented in Figures 23 and 24. These tests demonstrated a significant reduction of rotational behaviour in transplanted animals. Both the stepping adjustments and forelimb placing non-pharmacological tests also demonstrated a significant increase in mobility after stem cell therapy (Fig 25). In conclusion, the results of both, the pharmacological and non-pharmacological tests, demonstrated a significant reduction of Parkinsonism following transplantation of human ES cell-derived neural progenitors.

The fate of the transplanted human neural progenitors was studied by immunohistochemistry and RT-PCR. The sites of transplants were identified on H&E stained coronal sections. The human cells were found in the striatum along the injection tracts, identified by immunostainings for human specific mitochonodrial marker (Figure 26), human specific ribonuclear protein and nestin (Figure 27).

To evaluate the survival of the graft, sections were stained for human mitochondria. We compared the size of transplants at 24 hours and 3 months post transplantation. Since there was edema and free blood within and around the transplants at 24 hours, we measured the amount of human cells by quantifying the human-specific mitochondrial staining in low-power microscopic fields. This was calculated by multiplying the entire stained area with fluorescence intensity (above background). At the center of the transplant, the area of graft at 24 hours was 94+/-34 (arbitrary units; n=6) and at 12 weeks it

was 43+/-18 units (n=5). This indicates approximately 45% graft survival at 12 weeks after transplantation.

Given the potential of ES cells to generate teratomas after transplantation, we have evaluated the percentage of proliferating cells within the grafts. At 24 hours post transplantation, the majority of cells (64.5%) were in a proliferative state as indicated by positive PCNA and ki67 staining. At 12 weeks, there were very rare (<0.2%) PCNA+ cells (Fig. 28). Ki-67+ or PCNA+ cells were not observed in the host striatal parenchyma near the graft.. In addition, H&E stained sections, covering the entire brain did not reveal teratomas or any other tumor formation in transplanted rats.

Double staining with anti TH and anti human mitochondria at 12 weeks post-transplantation demonstrated that TH+ neurons were generated from the transplanted human cells (Fig 29). The number of TH+ fibers counted in human mitochondria+ areas, relative to number of DAPI+ counterstained nuclei, indicated that 0.41+/-0.3% of human cells generated TH+ fibers (n=9). If approximately $4 \times 10^5$ viable cells were transplanted into each rat, it may be estimated, therefore, that the transplants generated on average 740 TH+ neurons at 12 weeks post-transplantation.

To support the acquisition of a dopaminergic fate by the engrafted human progenitors, we have demonstrated by immunohistochemistry the expression of human DAT within the lesioned striatum 12 weeks after transplantation (Figure 29E). We have further demonstrated the expression of human specific transcripts of midbrain markers in brain samples from transplanted animals. Total RNA was extracted using the RNeasy kit (Qiagen) from midbrain samples that included the graft from stem cell (n=2) and vehicle transplanted (n=1) Parkinsonian rats. The RT-PCR reaction and the details of human specific primers are described above. Transcripts of human midbrain and dopaminergic neuron markers were expressed in samples from animals that received stem cell transplantation and were not detected in control animals (Figure 30).

In conclusion the results of these experiments demonstrate the long-term survival of hES cell-derived neural progenitors after transplantation to the striatum of parkinsonian rats. Proliferation of the transplanted cells decayed with time, teratoma tumor formation was not observed and the engrafted progenitors differentiated *in vivo* into DA neurons that led to functional recovery of Parkinsonism.

**Example 4: Transplantation of Human Embryonic Stem Cell - Derived Neural Progenitors Corrects Deficits in a Rat Parkinson Model**

Highly enriched cultures of neural progenitors from hES cells were grafted into the striatum of Parkinsonian rats. A significant fraction of the graft survived for at least 12 weeks, the transplanted cells stopped proliferating and teratoma tumors were not observed. The grafted cells differentiated *in vivo* into DA neurons though at prevalence (0.41%) similar to the one observed following spontaneous differentiation *in vitro*. Transplanted rats exhibited significant improvement in rotational behaviour that was induced by d-amphetamine and by apomorphine, and in stepping and placing non-pharmacological behavioural tests. Long-term survival of the grafted cells, lack of teratoma tumor formation, and the spontaneous differentiation of a fraction of the transplanted cells into DA neurons that reduced motor asymmetries and improved behavioural deficits of Parkinsonian rats was demonstrated. This study indicates the potential of hES cells to induce functional recovery in an animal model of Parkinson's disease.

A Development and characterization of hES cell-derived NPs

Differentiation of hES cells into highly enriched cultures of proliferating NPs was accomplished according to our simple two-step protocol (Reubinoff et al., 2001) with some modifications. In the first step, hES cell colonies (Figure 31A) were cultured for prolonged periods on feeders in the presence of the BMP antagonist noggin. Under these culture conditions, the hES cells in most of the colonies differentiated almost uniformly into tightly packed small progenitor cells. Briefly, human ES cells (HES-1 cell line, Reubinoff et al., 2000) with a stable normal (46XX) karyotype were cultured on mitomycin C treated mouse embryonic fibroblast feeder layer in gelatin- coated tissue culture dishes

(Figure.31A). To induce neural differentiation, clumps of undifferentiated hES cells were plated on fresh mitotically inactivated feeders and cultured for eight days in serum containing medium comprised of DMEM (Gibco, Gaithersburg, MD), containing glucose 4500mg/L without sodium pyruvate, supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), 0.1mM beta-mercaptoethanol, 1% non-essential amino acids, 2mM glutamine, 50u/ml penicillin, 50μg/ml streptomycin (Gibco) and 500ng/ml noggin (R&D Systems Inc., Minneapolis, MN). The medium was replaced every other day. Noggin was then omitted and the cells were further cultured in the same medium for additional 6 days. At this time, 70%-90% of the colonies differentiated almost uniformly into tightly packed small cells with a uniform gray opaque appearance under dark field stereomicroscopy (Figure 31B). In parallel, the colonies acquired a nearly uniform gray opaque appearance under dark field stereomicroscope (Figure 3B).

In the second step, patches containing about 150 cells each were cut out from the gray opaque areas, using a razor blade (surgical blade #15), and replated in serum-free medium that consisted of DMEM/F12 (1:1), B27 supplementation (1:50), glutamine 2 mM, penicillin 50 u/ml and streptomycin 50 μg/ml (Gibco), and supplemented with 20 ng/ml human recombinant epidermal growth factor (EGF), and 20 ng/ml basic fibroblast growth factor (bFGF) (R & D Systems Inc.). The clusters of cells developed into round spheres that were sub-cultured once a week as previously described (Reubinoff et al., 2001) (Figure 31C). The medium was replaced twice a week.

At this stage, prior to implantation into Parkinsonian rats, the phenotype of the cells within the spheres was characterized as well as their developmental potential to give rise to midbrain DA neurons. Indirect immunofluorescence analysis following disaggregation of the spheres demonstrated that >90% of the cells within the spheres expressed markers of neural progenitors (Figure 32J). Thus, the sphere cultures were highly enriched for neural progenitors.

The regulatory genes (*OTX1* and *OTX2, Pax2, Pax5, Wnt1, En1* and *En2)* and the transcription factors *Nurr1 and Lmx1b* were all expressed by the progenitors

within the spheres suggesting that these neural progenitors had the developmental potential to give rise to midbrain DA neurons (Figure 32). Some of the genes were also weakly expressed by cells within the undifferentiated hES cell cultures.

The phenotype of the neural progenitors following spontaneous differentiation *in vitro* has been characterised. Upon withdrawal of mitogens from the medium and plating on laminin, the spheres attached rapidly, and cells migrated out to form a monolayer of differentiated cells. The expression of transcripts of the regulatory genes of midbrain development and markers of DA neurons was up regulated in the differentiated progeny (Figure 32). After 7 days of differentiation, immunocytochemical studies were performed. Standard protocols were used for the immunophenotyping of disaggregated progenitor cells and differentiated cells following fixation with 4% paraformaldehyde. Primary antibodies localisation was performed by using swine anti-rabbit and goat anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (FITC) (Dako, A/S Denmark; 1:20-50), goat anti mouse IgM conjugated to FITC (Jackson Lab. West Grove, PA: 1:100), goat anti rabbit Ig conjugated to Texas Red (Jackson Lab., 1:100) and goat anti mouse IgG conjugated to $Cy^{TH}3$ (Jackson Lab., 1:500). Proper controls for primary and secondary antibodies revealed neither non-specific staining nor antibody cross reactivity.

To characterize the immunophenotype of cells within the aggregates, spheres that were cultivated for 6 weeks were mechanically partially disaggregated, and the resulting small clumps and single cells were plated in serum-free medium, as described above, on poly-D-lysine (30-70 kDa, 10 μg/ml, Sigma, St. Louis, MO) and laminin (4 μg/ml, Sigma). The cells were fixed after 24 hours and examined for the expression of N-CAM (Dako; mouse IgG 1:10), nestin (rabbit antiserum a kind gift of Dr. Ron McKay; 1:25; or from Chemicon, Temecula, CA; rabbit anti human 1:100-200), A2B5 (ATCC, Manassas, VA; mouse clone 105 1:20), PSA-N-CAM (Developmental Studies Hybridoma Bank, Iowa city, IA; mouse undiluted, or from Chemicon Temecula, CA mouse 1:200). One-to-two hundred cells were scored within random fields (at X 400) for the expression of each of these markers and the experiments were repeated at least 3 times.

To induce differentiation, spheres that were 6 weeks in culture, were disaggregated into small clumps and plated on poly-D-lysine and laminin in serum-free growth medium (as described above) without supplementation of growth factors for 1 week. Differentiated cells were analysed for the expression of GFAP (Dako; rabbit Ig 1:400), β-tubulin III (Sigma; mouse IgG 1:2000), serotonin (Sigma; rabbit 1:1000) and tyrosine hydroxylase (TH) (Pel-Freez anti Rabbit polyclonal, 1:100). To determine the percentage of neurons, 200-500 cells were scored within random fields of the outgrowth from differentiating clumps (at X400) and the experiments were repeated at least 3 times.

From the immunocytochemical studies, it was found that 30% of the cells were immunoreactive with anti β-tubulin III (a neuronal marker) (Fig. 31J, H and I). Double labelling studies showed that about 0.5% of the cells in these cultures co-expressed β-tubulin III and tyrosine hydroxylase (TH) (Figure 31H), and 0.8-1% co-expressed β-tubulin III and serotonin (Figure 31I). These results suggested that under our culture conditions a low percentage (<1%) of the progenitors spontaneously differentiated *in vitro* into putative mid/hind brain neurons.

<u>B Survival and differentiation of human NPs after transplantation to Parkinsonian rats</u>

The survival, differentiation and function of the hES cell-derived NPs *in vivo* after transplantation to the rat animal model of Parkinson's disease was ananlysed.

A Parkinson's disease model was induced in adult Sprague-Dawley rats by stereotaxic injection of the neurotoxin 6-hydroxydopamine to cause unilateral nigrostriatal lesions. 8μg/rat of 6-hydroxydopamine were injected in 4μl into the right Substantia Nigra. 6-hydroxydopamine was injected to the right substantia-nigra to deplete dopaminergic innervation in the ipsilateral striatum. Coordinates of injection were P=4.8, L=1.7, H=-8.6. Preliminary experiments confirmed this resulted in the complete loss of TH+ stained neurons in the ipsilateral striatum, whereas TH expression in the contra-lateral side was preserved.

At 18 days after the lesion, Parkinsonian rats with >350 rotations per hour after S.C. injection of apomorphine (25mg/100gr body weight) were selected for the transplantation experiment.

At 3 weeks after the lesion, hES-cell derived neural spheres that had been passaged for 6 weeks were grafted (along two tracts, 4x $10^5$ cells/animal) into the right striatum of rats that were pre-selected for apomorphine – induced high rotational activity. Two days after selection of Parkinsonian rats, animals were stereotaxically injected with either neurospheres or medium into 2 sites of the right striatum. The hES - derived neural spheres (passaged for 6 weeks) were mechanically dissociated into small clumps and transplanted (about 400,000cells in 12-14μl/animal) with a hamilton syringe, along 2 tracts per striatum, using a stereotaxic device. Coordinates for transplantation were: anteromedial tract - A-P= 0, L= 3.5, H= -7.5 to -4.5 and posterolateral tract - A= 1, L= 2, H= -7.5 to -4. Control rats underwent sham operation and were injected with vehicle solution. To prevent rejection of grafted human cells, all rats (transplanted and controls) received daily I.P injection of 10mg/kg cyclosporine A (Sandimmune, Sandoz).

The rats were sacrificed for histopathological analysis of the graft 24 hours after transplantation (n=6), and after behavioral follow up of 12 weeks (21 sphere and 17 vehicle grafted rats).

At the end of follow-up and behavioral studies, rats were euthanized by pentobarbital overdose and perfused with saline and 4% paraformaldehyde. Serial 8μm coronal frozen sections were prepared and every seventh section was stained with hematoxylin and eosin (H&E) to identify the graft in the brain. In areas in which a graft was identified, the sections were post fixated with 4% formaldehyde. Immunofluorescent stainings were performed with the following primary antibodies; human-specific mitochondrial antibody (mouse IgG, Chemicon; 1:20), tyrosine hydoxylase (TH rabbit IgG, Chemicon 1:100), nestin (antibody as detailed above; 1:50), neurofilament heavy chain (NF-200 mouse

IgG, Sigma; 1:100), β III-tubulin (antibody as detailed above; 1:50), neuronal nuclei marker (NeuN mouse IgG Chemicon 1:50 ).

Sections that were post fixated with acetone were stained with human-specific ribonuclear protein antibody (RNP, mouse IgM, Chemicon; 1:20). Sections that were post fixated with methanol were stained with Proliferating Cell Nuclear Antigen (PCNA mouse IgG Chemicon 1:100), Ki67 Antigen (Rabbit polyclonal, Novocastra laboratories 1:100), vesicular monoamine transporter 2 (VMAT2, rabbit, Pel-Freez 1:50-100), human dopamine transporter (rat monoclonal, Chemicon 1:2000). Goat anti mouse IgG conjugated to Alexa 488 or Cy3, goat anti mouse IgM conjugated to texas red, goat anti rabbit IgG conjugated to Alexa 488 or to texas red (Jackson; 1:100) and goat anti rat IgG conjugated to Alexa 488 (Molecular Probes 1:500) were used where appropriate for detection of primary antibodies. Double stains were performed by using primary antibodies of different species or Ig subtype, followed by non-cross-reactive secondary antibodies. Double labeling for TH and human mitochondria was used to evaluate the percentage of TH+ neurons within the grafts. At least 3 high power microscopic fields per section and 3 sections per animal were counted for TH+ cells within the graft. Images were taken by a fluorescent microscope (Nikon E600) or confocal microscope (Zeiss), using channels for Alexa 488 fluorescence, Cy3 and Cy5 fluorescence and Nomarsky optics.

The grafts were easily identified on H&E stained sections and by fluorescent DAPI nuclear counterstaining. At 12 weeks after transplantation, a graft was found in 17 animals. In each of these animals, two grafts were found, most often as a tubular mass of cells along the needle tract within the striatum. In five animals one of the two grafts was ectopic and was observed as a round mass in the cortex.

Anti human-specific mitochondria antibodies to specifically identify human cells in transplanted rat brain sections (Figure 33A) were used. Identification of human cells was confirmed by staining for human-specific ribo-nuclear protein (Figure 33B). At 24 hours post-transplantation, there was widespread expression of nestin in the graft (Figure 33C). At 12 weeks post-transplantation,

the positive human mitochondria cells were found only at the site of transplantation, and there was no indication for cell migration to neighboring regions.

To evaluate graft survival, estimations of the number of transplanted cells at 24 hours and 3 months post- transplantation were compared. Since actual counting of the grafted cells was not practical an approach that allowed rough estimation and comparison of the number of cells within the grafts was used. A coronal section along and through the center of the tubular transplants was identified and chosen from serial H&E stained coronal sections. Assuming that the graft had a symmetrical tubular structure, the area of the graft in the section was proportional to the volume of the graft. Since there was edema and some free blood within and around the transplants at 24 hours, and the density of human cells differed between grafts, the area of the grafts in the selected coronal sections was not representative of the number of cells. To overcome this problem an adjacent section was stained for human mitochondria and the amount of human cells by quantifying the human-specific mitochondrial staining was measured. This was calculated by multiplying the entire stained area (in low-power microscopic fields) with fluorescence intensity above background. Twenty-four hours after transplantation, the overall graft mitochondrial staining in sections through the center of the transplants was 94+/-34 (arbitrary units; n=6) and at 12 weeks it was 43+/-18 units (n=5). This indicated approximately 45% graft survival at 12 weeks after transplantation.

Double staining with human specific markers and neuronal markers, indicated the generation of human mitochondria+, neurofilament+ (Figure 33D) and human ribonuclear protein+, Neun+ (Figure 33E) neurons from transplanted cells. Double staining for human mitochondria and tyrosine-hydroxylase (TH) showed the presence of graft-derived TH+ cells and fibers (Figure 33F-H). The vehicle-grafted animals showed no TH staining in the ipsilateral substantia nigra or the striatum. At 12 weeks post-transplantation, the number of TH+ cells in the human mitochondria stained areas, relative to the number of DAPI+ counterstained nuclei, indicated that 0.41+/-0.3% of the human cells generated TH+ neurons (n=9 animals). Since $4x10^5$ cells were transplanted into each rat, it

may be estimated, therefore, that the graft generated approximately 740 TH+ neurons.

Cells that were decorated with an antibody directed against human dopamine transporter were identified within the graft and were undetectable in the striatum of medium-grafted controls (Figure 33I). In addition, an antibody directed against human vesicular monoamine transporter (V-MAT2), a dopaminergic neuronal marker (Miller et al., 1999), decorated graft-derived cells that co-labeled with the human anti mitochondrial antibody (Figure 33J,K). V-MAT2+ cells were not observed in the striatum of sham operated animals.

To confirm the expression of human dopaminergic neuronal markers in the transplanted brains, RT-PCR analysis of striatal samples from vehicle and neural progenitor grafted rats was performed (as in Example 3). Transcripts of human midbrain and dopaminergic neuron markers were expressed in samples from animals that received stem cell transplantation (n= 3) and were not detected in vehicle-transplanted animals (n= 2). The expression of these human-specific mRNA transcripts was found only in the transplanted side, and not in the non-lesioned side of the same animals (Figure 34).

Given the potential of ES cells to generate teratomas after transplantation, we evaluated the percentage of proliferating cells within the grafts. At 24 hours post- transplantation, the majority of cells (64.5%) were in a proliferative state as indicated by positive PCNA (Figure 33L) and ki67 staining (not shown). At 12 weeks, there were very rare (<0.2%) PCNA+ cells (Figure 33M). In addition, H&E stained sections covering the entire brain, did not reveal teratomas or any other tumor formation in transplanted rats.

<u>C. Functional recovery in Parkinsonian rats after transplantation of hES-derived neural spheres.</u>

At 2 weeks, 1 month, 2 months, and 3 months after transplantation, the severity of the disease was scored by pharmacological tests and compared between hES cell transplanted and vehicle transplanted animals. Rotations were counted for 1 hour after S.C. injection of apomorphine (25μg/100g body weight) and for

1 hour after I.P. d-amphetamine (4mg/kg, performed 2 days later) by a computerized rotometer system (San-Diego Instruments,Inc).

Pharmacological-induced rotational behavior was measured in rats that were transplanted with spheres or with medium at 2 weeks (Baseline), 4 weeks, 8 weeks and 12 weeks after engraftment (Figure 35). The 2 rats in which no graft was found did not exhibit any improvement in motor function. In transplanted rats (n=10 rats in which a graft was found), amphetamine-induced rotations decreased from 607±200/hour at baseline to 334±130/hour at 12 weeks (45% decrease, p=0.001, Figure 35A). It should be noted that the difference was already statistically significant at 8 weeks. Amphetamine-induced rotations increased in the control group (n=10 rats) from 480±210/hour to 571±235/hour at 12 weeks. Apomorphine-induced rotations decreased in the transplanted group (n=19 rats) from an average of 624±220/hour at baseline to 423±158 rotations/ hour at 12 weeks (31% decrease, p=0.0015, Figure 35B). The difference between the groups was significant already at 8 weeks. The control group (n=17) rotated 567±169 times per hour at baseline and 571±112 after 12 weeks.

Non-pharmacological tests were performed at 2 weeks and 3 months after transplantation. These included stepping adjustments (Olsson et al., 1995) and forelimb placing (Lindner et al., 1997) tests. The number of stepping adjustments was counted for each forelimb during slow-sideway movements in forehand and backhand directions over a standard flat surface. The stepping adjustments test was repeated three times for each forelimb during three consecutive days. The forelimb-placing test assesses the rats' ability to make directed forelimb movements in response to a sensory stimulus. Rats were held with their limbs hanging unsupported. They were then raised to the side of a table so that their whiskers made contact with the top surface while the length of their body paralleled the edge of the tabletop. Normally, rats place their forelimb on the tabletop almost every time. Each test included ten trials of placing of each forelimb and was repeated in three consecutive days. The results of both tests are expressed as percentage of forelimb stepping adjustments and placing in the lesioned side as compared to the non-lesioned side. The mean number

of rotations and the mean results (in percentage) of non-pharmacological tests were compared between the experimental groups using the student t-test.

The behavioral analysis was extended to include also the stepping adjustments (Olsson *et al.*, 1995) and forelimb placing (Lindner *et al* 1997) non-pharmacological tests. Non-pharmacological tests provide a more direct measure of motor deficits analogous to those found in human Parkinson's disease (Kim et al., 2002). Stepping and placing were examined at baseline (2 weeks) and at 12 weeks after transplantation. At 2 weeks the transplanted rats did not make any stepping or placing in the lesioned side. At 12 weeks there was a significant improvement in both non-pharmacological tests as compared to baseline and to control rats (Figure 35 C, D).

Values in the behavioural tests are given as mean± standard error. Statistical analysis for the pharmacological tests was performed by one-tailed analysis of variance (ANOVA), followed by Bonferroni post hoc test. In the non-pharmacological tests the groups were compared by the student's t-test. A statistical significant difference was considered when $p<0.05$.

This study shows that transplantation of hES cell-derived neural spheres improves the motor function in rats in an experimental model of Parkinson's disease.

A simple two-step protocol to direct the differentiation of hES cells *in-vitro* into highly enriched cultures of proliferating NPs has been used. The NPs expressed transcripts of key regulatory genes of midbrain development as well as markers of dopaminergic neurons supporting their potential to differentiate into midbrain dopaminergic neurons. Following transplantation, a significant clinical effect was evident by all four different behavioral tests. Functional recovery in the pharmacological behavioral tests was evident and significant at 8 and 12 weeks after transplantation. The functional recovery in the lesioned rats was correlated by the demonstration of transplant-derived dopaminergic cells, as indicated by immunofluorescent stainings and RT-PCR. At the RNA level, human specific transcripts of key regulatory genes of midbrain

development as well as markers of DA neurons were observed in brain samples from the location of the graft. At the protein level, human cells decorated with antibodies against dopamine neuron specific markers including DAT (Kim et al 2002) and VMAT2 (Miller et al., 1999) were observed within the grafts. Collectively this data suggest that the behavioural recovery that we have observed was related to the differentiation of the grafted NPs into functional DA neurons. However, further studies are required to confirm that hES cells can differentiate into DA neurons with phenotype, function and interaction with host neurons that are identical to those of authentic midbrain DA neurons.

These findings suggest that commitment of the human cells to a dopaminergic fate prior to their transplantation is a pre-requisite to obtain a larger number of graft-derived dopaminergic neurons.

Transplantation of low dose of undifferentiated mouse ES cells into parkinsonian rats resulted in the formation of teratomas in a high percentage of animals (Bjorklund et al., 2002). Here, the human ES cells were directed to differentiate *in-vitro* into neural progenitors prior to transplantation and ceased to express the transcription factor Oct-4, a marker of undifferentiated ES cells. The human grafts did not produce teratomas or non-neural tissue in the rat brains. In correlation, the transplanted cells ceased to express markers of proliferating cells. Nevertheless, additional extensive long-term studies are required to determine the safety of human ES derived neural progeny transplantation and to rule out potential hazards such as tumor formation or the development of cells from other lineages.

This study shows for the first time that human ES cell-derived NPs can induce functional recovery in an experimental model of Parkinson's disease. The therapeutic effect, demonstrated in this study, indicate the potential of hES cells for transplantation therapy and encourage further efforts that may eventually allow the use of hES cells for the treatment of neurological disorders.

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Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

## CLAIMS

1. A method of directing the fate of human embryonic stem cells towards neural progenitor cells *in vitro* said method including the steps of:

culturing undifferentiated human ES cells in a defined serum free medium that contains FGF-2 and an antagonist of bone morphogenic proteins (BMP).

2. A method according to claim 1 wherein the BMP antagonist is a direct antagonist of BMP or a ligand binding domain from a BMP receptor.

3. A method according to claim 1 or 2 wherein the BMP antagonist is selected from the group including fetuin, noggin, chordin, gremlin, follistatin, Cerberus, amnionless, DAN or the ectodomain of BMRIA (a BMP receptor protein).

4. A method according to any one of claims 1 to 3 wherein the BMP antagonist is noggin.

5. A method according to any one of claims 1 to 4 wherein the noggin is in the range of 350 to 700ng/ml.

6. A method according to any one of claims 1 to 5 wherein FGF-2 is approximately 20 ng/ml.

7. A cell culture comprising neural progenitor cells differentiated from hES cells and wherein the neural progenitors are non-committed to a neural fate.

8. A cell culture according to claim 7 wherein expression of early neural markers increases over 3 weeks when cultured in NPM-FGF-2 compared to culturing in KO medium.

9. A cell culture according to claim 8 wherein the early neural markers are selected from the group including nestin, A2B5, N-CAM, PSA-NCAM and β-tubulin III.

10. A cell culture according to claim 8 or 9 wherein FGF-2 is approximately 20 ng/ml.

11. A cell culture according to any one of claims 7 to 10 wherein the expression of early neural markers is further increased by culturing in the presence of a BMP antagonist.

12. A cell culture according to claim 11 wherein the BMP antagonist is selected from the group including fetuin, noggin, chordin, gremlin, follistatin, Cerberus, amnionless, DAN or the ectodomain of BMRIA (a BMP receptor protein).

13. A cell culture according to claim 12 wherein the BMP antagonist is noggin.

14. A cell culture according to claim 13 wherein noggin is in the range of 350 to 700 ng/ml.

15. A cell culture according to any one of claims 11 to 14 wherein at least 75% of the cells have an increased expression of early neural cell markers selected from the group including nestin, A2B5, N-CAM, PSA-NCAM and β-tubulin III.

16. A cell culture according to any one of claims 11 to 15 wherein at least 95 to 100% of the cells have an increased expression of early neural cell markers selected from the group including nestin, A2B5, N-CAM, PSA-NCAM and β-tubulin III.

17. A cell culture according to any one of claims 11 to 16 wherein A2B5 expression is increased in at least 95% of the cells in culture.

18. A cell culture according to any one of claims 11 to 17 wherein NCAM expression is increased in at least 73% of the cells in culture.

19. A cell culture according to any one of claims 7 to 18 wherein expression of non-neural markers selected from the group including alpha-fetal protein, the endodermal marker HNF3α or the epidermal marker keratin-14 is reduced.

20. A cell culture according to any one of claims 7 to 19 wherein the neural progenitor cells over express *Nurr-1*.

21. A cell culture according to claim 20 wherein the neural progenitor cells co express *Nurr-1* with TH.

22. An isolated neural progenitor cell differentiated from a hES cell and wherein said neural progenitor is not committed to a neural fate.

23. An isolated neural progenitor cell derived form a cell culture according to any one of claims 7 to 21.

24. A method of directing neural fate in a human embryonic stem (hES) cell *in vitro* said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and

culturing the neural progenitor cell in the presence of a neural fate inducer selected from the group including at least one of Fibroblast Growth Factor (FGF), Sonic Hedgehog Protein (SHH), cAMP inducers, Protein Kinase C (PKC) inducers, dopamine and ascorbic acid (AA) or any combination thereof.

25. A method according to claim 24 wherein the neural fate is a neuronal cell type.

26. A method according to claim 25 wherein the neuronal cell type is selected from the group including hES derived GABAergic, glutmertegic,

cholinergic, motor neurones, dopaminergic and serotonergic neurons or tyrosine-hydroxylase (TH) positive cells.

27. A method according to any one of claims 24 to 26 wherein the neural fate is a midbrain neural fate.

28. A method according to any one of claims 24 to 27 wherein the neural progenitor cell from the hES cell culture has been treated with a BMP antagonist.

29. A method according to claim 28 wherein the BMP antagonist is noggin.

30. A method according to claim 28 wherein noggin is in the range of 350 to 700 ng/ml.

31. A method according to any one of claims 28 to 30 wherein the neural progenitor cells are cultured with the presence of FGF with/without EGF and/or LIF.

32. A method according to claim 31 wherein FGF is approximately 20 ng/ml, EGF is approximately 20 ng/ml and LIF is approximately 10 ng/ml.

33. A method to block differentiation of hES cells towards non-neural lineages, said method comprising

culturing undifferentiated hES cells in the presence of a BMP antagonist;

removing the BMP antagonist from culture; and

reculturing the hES cells in the presence of NPM and FGF without the BMP antagonist.

34. A method according to claim 33 wherein the BMP antagonist is noggin.

35. A method according to claim 34 wherein noggin is in the range of 350 to 700 ng/ml.

36. A method according to any one of claims 24 to 35 wherein FGF is selected from the group including FGF-1, FGF-2, FGF-6, FGF-8, FGF-9, FGF-98 and FGF-17 or any biologically active fragment or mutein thereof.

37. A method according to any one of claims 24 to 36 wherein the FGF is selected from the group including FGF-1, FGF-8 or FGF-17 alone or in combination.

38. A method of directing midbrain fate to a hES cell *in vitro*, said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and

culturing the neural progenitor cell in the presence of a midbrain fate inducer selected from the group including any one of FGF-1, FGF-8, FGF-17, SHH, AA, cAMP inducers, PKC inducers and dopamine or any combination thereof.

39. A method according to claim 38 wherein the neural progenitor cell from the hES cell culture has been pre-cultured in the presence of FGF-2, with/without EGF and/or LIF.

40. A method according to claim 39 wherein the FGF-2, EGF and LIF are removed before culturing the pre-cultured neural progenitor cell in the presence of the mid-brain fate inducer.

41. A method according to any one of claims 38 to 40 wherein the FGF-1, FGF-8 or FGF-17 is present at a concentration of approximately 100 to 200ng/ml.

42. A method according to anyone of claims 38 to 41 wherein SHH is present in combination with other midbrain inducers at a concentration of about 0.5 to 1 $\mu$g/ml.

43. A method according to any one of claims 38 to 42 wherein the CAMP inducer is present at a concentration of 0.25mm to 50μm in combination with other midbrain inducers.

44. A method according to any one of claims 38 to 43 wherein the PKC inducer is approximately 20μm in combination with other midbrain inducers.

45. A method according to any one of claims 38 to 44 wherein the midbrain fate inducers are present as a combination of any one of the following combinations:

(i) FGF-1, FGF-8, FGF-17, SHH,IBMX, forskolin, PMA/TPA and dopamine; or
(ii) FGF-8 and SHH, IBMX, forskolin, PMA/TPA and dopamine; or
(iii) FGF-17 and SHH, IBMX, forskolin, PMA/TPA and dopamine; or
(iv) FGF-1, and IBMX, forskolin, PMA/TPA and dopamine; or
(v) FGF-8 alone; or
(vi) FGF-17 alone; or
(vii) IBMX, forskolin, PMA/TPA, and dopamine.

46. A method according to anyone of claims 38 to 45 further including culturing the neural progenitor cell in the presence of AA or an analogue thereof.

47. A method according to claim 46 wherein the AA or analogue thereof is in the range of 400 to 800μm.

48. A method according to any one of claims 38 to 47 further including culturing the neural progenitor cell in the presence of NT4 or an equivalent thereof.

49. A method according to claim 48 wherein NT4 is at concentration of about 20ng/ml.

50. A method according to any one of claims 38 to 49 further including culturing the neural progenitor cells on poly-D-lysine and laminin.

51. A method according to claim 50 wherein the poly-D-lysine is in the range of 5 to 15 ng/ml and laminin is in the range of 1 to 10 μg/ml.

52. A method according to claim 50 or 51 wherein the concentration of poly-D-lysine is about 10μg/ml and laminin is about 4μg/ml.

53. A cell culture composition comprising neural progenitors with a committed neural fate wherein said neural progenitors are prepared by a method according to any one of claims 24 to 52.

54. A cell culture according to claim 53 wherein the neural fate is a midbrain neural fate.

55. A cell culture comprising clumps of differentiated hES cells and wherein at least 30% of the clumps include a significant number (>50) of neurons with a midbrain fate.

56. A cell culture according to claim 55 wherein at least 60% of the clumps include a significant number (>50) of neurons with a midbrain fate.

57. A cell culture according to any one of claims 53 to 56 which produces $TH^+$ neurons upon differentiation.

58. A cell culture according to any one of claims 53 to 56 which produces dopaminergic neurons upon differentiation.

59. A cell culture comprising clumps of differentiated hES cells and wherein at least 30% of the neurons (β-tubulin III+ cells) express TH.

60. A cell culture composition according to claim 58 wherein at least 60% of the neurons (β-tubulin III+ cells) express TH.

61. A cell culture comprising a population of differentiated hES cells wherein the population is substantially neural progenitors prepared by a method according to any one of claims 24 to 52 said neural progenitor having a midbrain fate.

62. A cell culture according to claim 61 wherein the neural progenitors give rise to neurons that are TH+ or dopaminergic upon differentiation.

63. A cell culture according to claim 61, wherein the neural progenitors give rise to neurons that are dopaminergic upon differentiation.

64. An isolated human neural progenitor cell having a committed neural fate.

65. An isolated human neural progenitor cell according to claim 64 having a committed midbrain fate.

66. An isolated human neural progenitor cell according to claim 64 or 65 which can differentiate into a TH+ neuron.

67. An isolated human neural progenitor cell according to claim 64 or 65 which can differentiate into a dopaminergic neuron.

68. An isolated human neural progenitor cell having a committed neural fate.

69. An isolated human neural progenitor cell according to claim 68 having a committed midbrain fate.

70. An isolated human neural progenitor cell according to claim 68 or 69 which can differentiate into a TH+ neuron.

71. An isolated human neural progenitor cell according to claim 68 or 69 which can differentiate into a dopaminergic neuron.

72. An isolated human dopaminergic neuron derived from a hES cell which has been directed to a neural fate by a method according to any one of claims 24 to 52.

73. An isolated human TH+ neuron derived from a hES cell which has been directed to a neural fate by a method according to any one of claims 24 to 52.

74. A human neural fate inducer composition for inducing neural fate in a cultured hES cell, said composition comprising a neural fate inducer selected from the group including Fibroblast Growth Factor (FGF), ascorbic acid (AA), Sonic Hedgehog Protein (SHH), cAMP inducers, Protein Kinase C (PKC) inducers and dopamine or any combination thereof.

75. A method of treating a neurological condition in an animal, said method comprising administering an effective amount of *in vitro* derived neural progenitor cells to the animal.

76. A method of treating a neurological condition in an animal, said method comprising administering an effective amount of *in vitro* derived neural progenitor cells to the animal wherein the neural progenitor cell is prepared by a method according to any one of claims 24 to 52.

77. A method according to claim 75 or 76 wherein the neurological disorder is selected from the group including Alzheimer's disease, Parkinson's disease, Lewy body dementia, multiple sclerosis, epilepsy, cerebellar ataxia, progressive supranuclear palsy, multi-system atrophies, spino-cerebelar degenerations, optic nerve and retinal diseases including retinal and macular degeneration and retinitis pigmentosa, amyotrophic lateral sclerosis, affective disorders, anxiety disorders, obsessive compulsive disorders, personality disorders, attention deficit disorder, attention deficit hyperactivity disorder, Tourette Syndrome, Tay Sachs, Nieman Pick, and other lipid storage and genetic brain diseases and/or schizophrenia, subjects suffering from or at risk for nerve damage from cerebrovascular disorders such as stroke in the brain or spinal cord, from CNS

infections including meningitis and HIV from tumors of the brain and spinal cord, or from a prion disease.

78. A method according to claim 77 wherein the condition is Parkinson's disease.

79. A method according to any one of claims 75 to 78 wherein the neural progenitor cell is delivered to the basal ganglia, substantia nigra, or the locus ceruleus, brain, parenchyma of the brain, or spaces containing cerebrospinal fluids.

80. A method of directing neural fate in a human embryonic stem (hES) cell *in vitro* said method comprising the steps of:

obtaining a neural progenitor cell from a hES cell culture; and

inducing an overexpression of *Nurr* 1 and/or Lmx1b in the hES cell.

81. A method according to claim 80 wherein overexpression occurs during a differentiation stage or during transplantation.

82. A method of enhancing the survival of transplanted DA neurons said method comprising

obtaining a neural progenitor cell from a hES cell culture or a cell differentiated from the neural progenitor cell; and

inducing an expression of GDNF and/or BDNF in the neural progenitor cell or the cell differentiated from the neural progenitor.

83. A method of enhancing the survival of transplanted DA neurons said method comprising

obtaining a neural progenitor cell from a hES cell culture or a cell differentiated from the neural progenitor according to anyone of claims 22, 23, or 64 to 73; and

inducing an expression of GDNF and/or BDNF in the neural progenitor cell or the cell differentiated from the neural progenitor.

84. A method according to claim 82 or 83 wherein the expression of GDNF and/or BDNF is an overexpression.

85. A method according to anyone of claims 82 to 84 wherein the expression of the GDNF and/or the BDNF is induced when the cells are transplanted or during a differentiation stage.

86. A method of enhancing the survival of transplanted DA neurons said method comprising

obtaining a neural progenitor cell from a hES cell culture or a cell differentiated from the neural progenitor cell; and

inducing an expression of *Nurr-1* and/or Lmx 1b and GDNF and/or BDNF in the neural progenitor cell or the cell differentiated from the neural progenitor.

87. A method according to claim 86 wherein the expression of *Nurr-1* and/or Lmx 1b and GDNF and/or BDNF in the neural progenitor cell or the cell differentiated from the neural progenitor is an over-expression.

88. A method according to claim 86 or 87 wherein the expression occurs during a differentiation stage or upon transplantation.

89. A genetically modified cell prepared by the method according to anyone of claims 80, to 88.

90. A method of a treating neural condition in an animal, said method comprising transplanting the genetically modified hES cell according to claim 89 into the animal: and

inducing the expression of the *Nurr1* and/or Lmx 1b gene and/or the GDNF and/or BDNF survival factors in the cell.

91. A method according to claim 90 wherein the neural condition is Parkinson's disease.

92. A method according to claim 90 or 91 wherein the cell is transplanted to the basal ganglia, substantia nigra, or the locus ceruleus, brain, parenchyma of the brain, or spaces containing cerebrospinal fluids.

**Fig 1**

A B

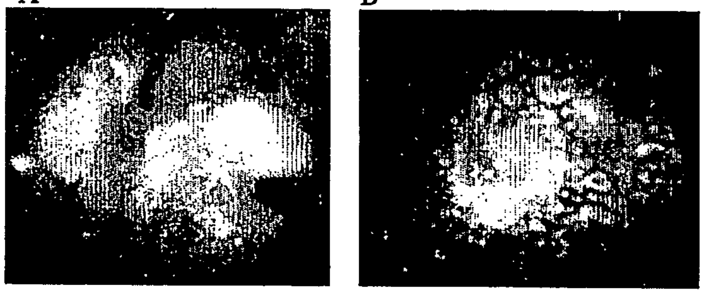


**Fig 2**

A B

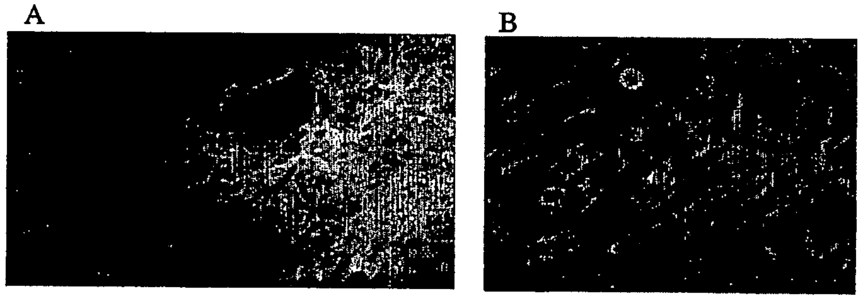


**Fig 3**

A B C D

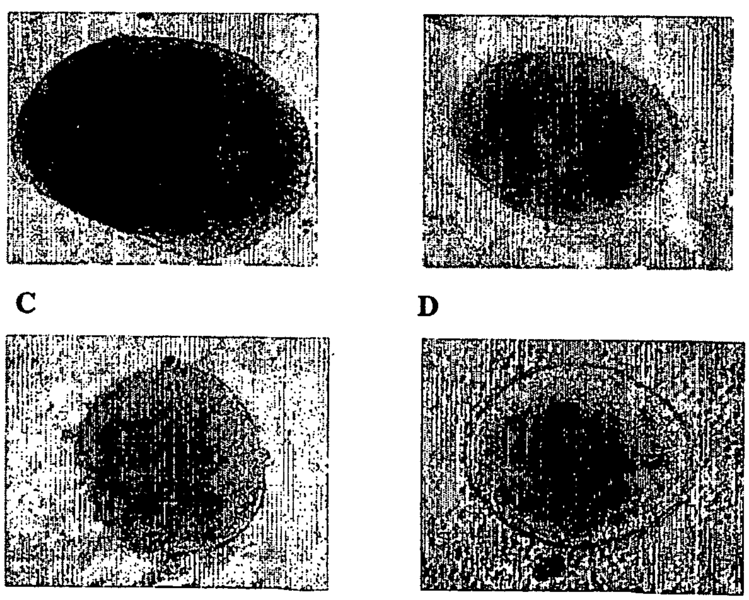
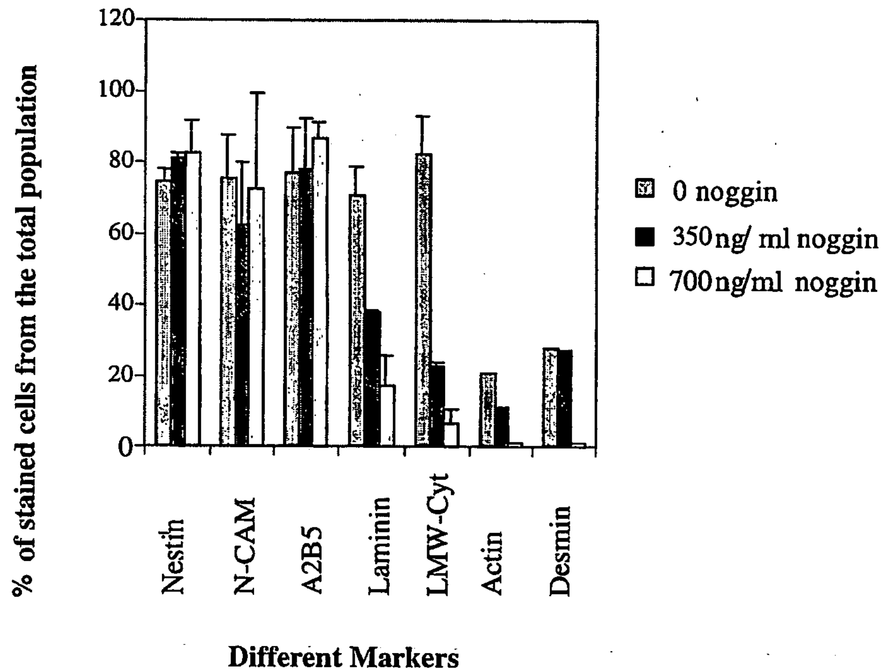


Fig 4


% of stained cells from the total population
120
100
80
60
40
20
0
Nestin
N-CAM
A2B5
Laminin
LMW-Cyt
Actin
Desmin
Different Markers
0 noggin
350ng/ ml noggin
700ng/ml noggin

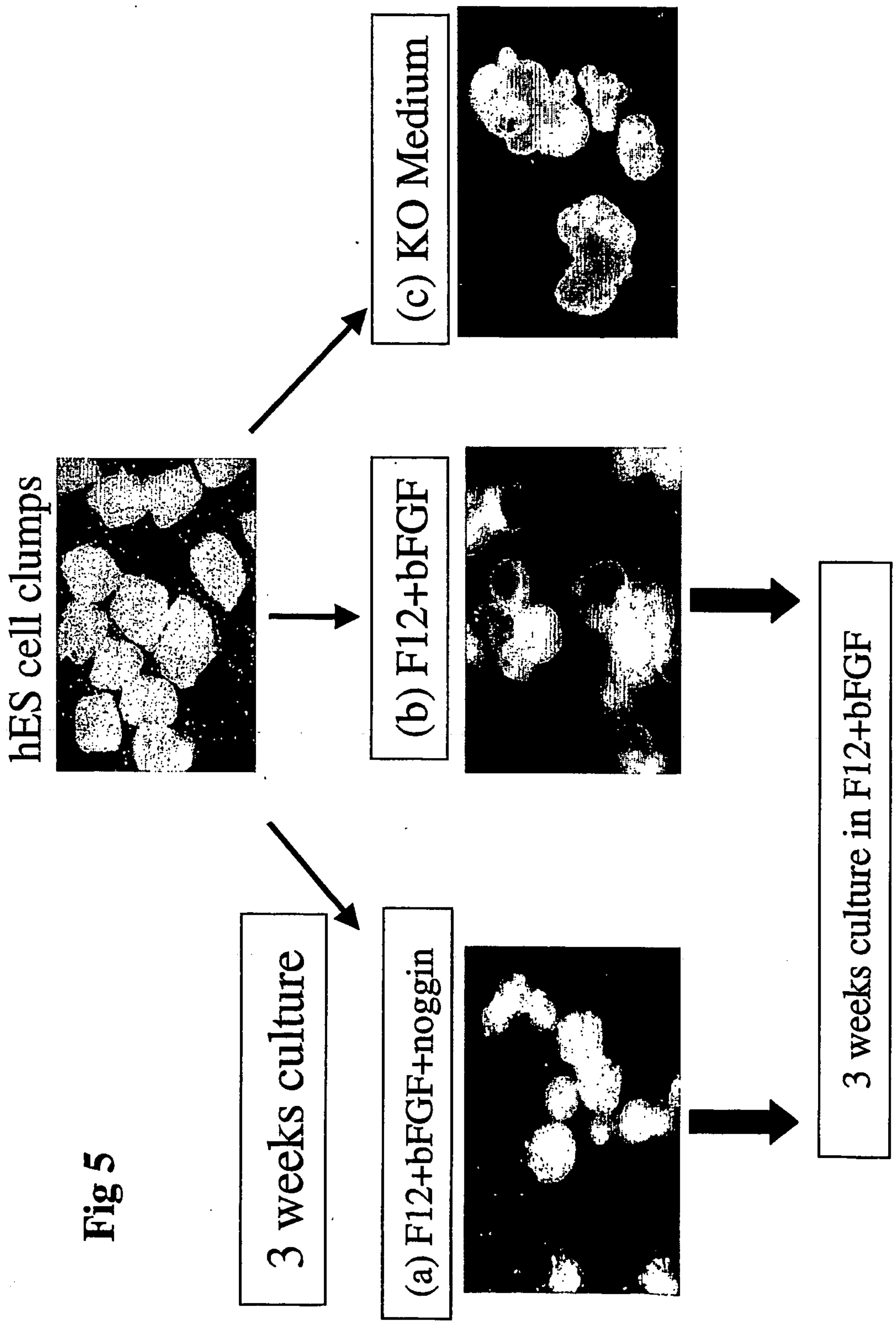

hES cell clumps
(c) KO Medium
(b) F12+bFGF
(a) F12+bFGF+noggin
3 weeks culture
3 weeks culture in F12+bFGF

Fig 5

**Fig.6**

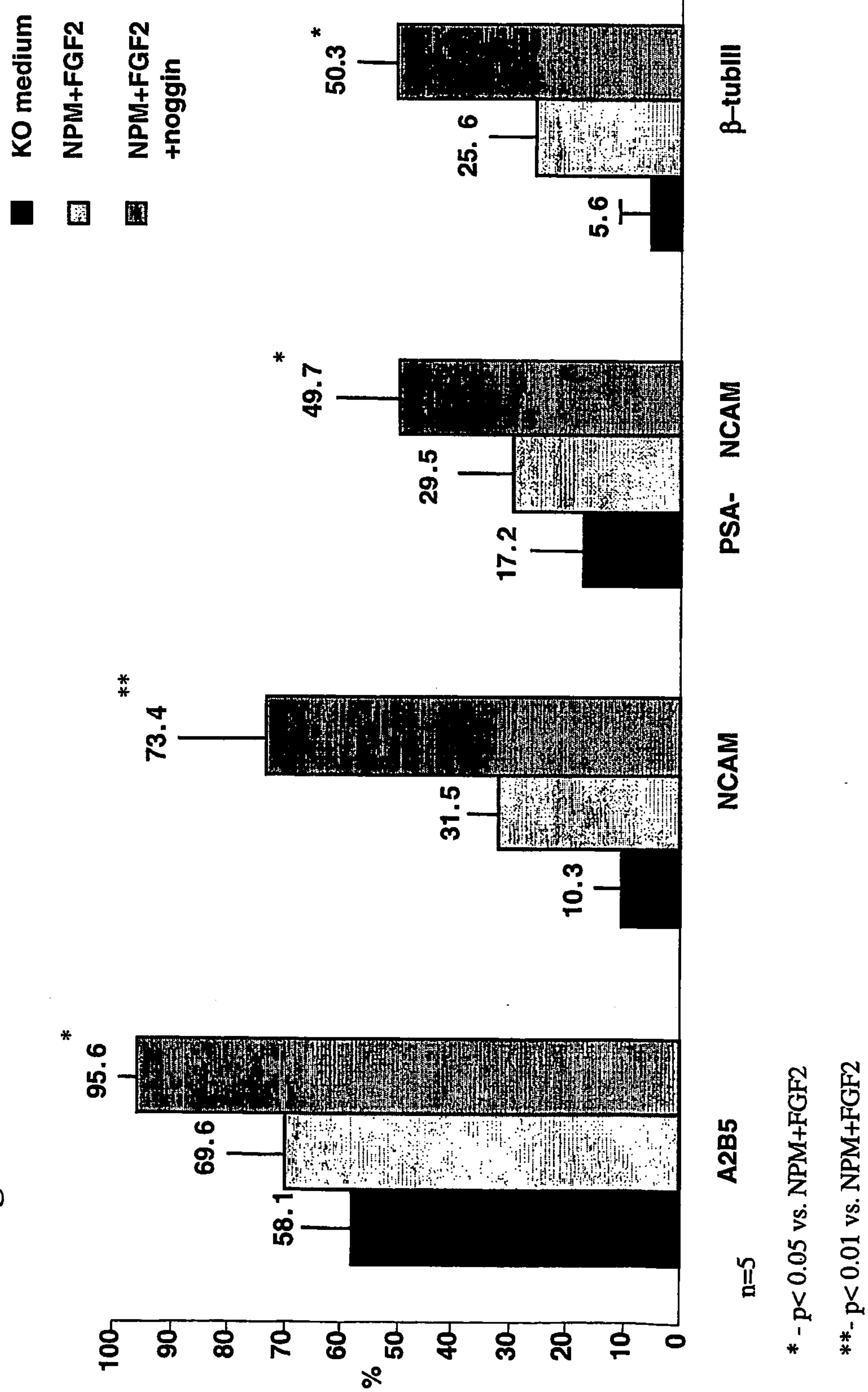

KO medium
NPM+FGF2
NPM+FGF2 +noggin
%
100
90
80
70
60
50
40
30
20
10
0
58.1
69.6
95.6
*
10.3
31.5
73.4
**
17.2
29.5
49.7
*
5.6
25. 6
50.3
*
A2B5
NCAM
PSA- NCAM
β–tubIII
n=5
* - p< 0.05 vs. NPM+FGF2
**- p< 0.01 vs. NPM+FGF2

Fig.7

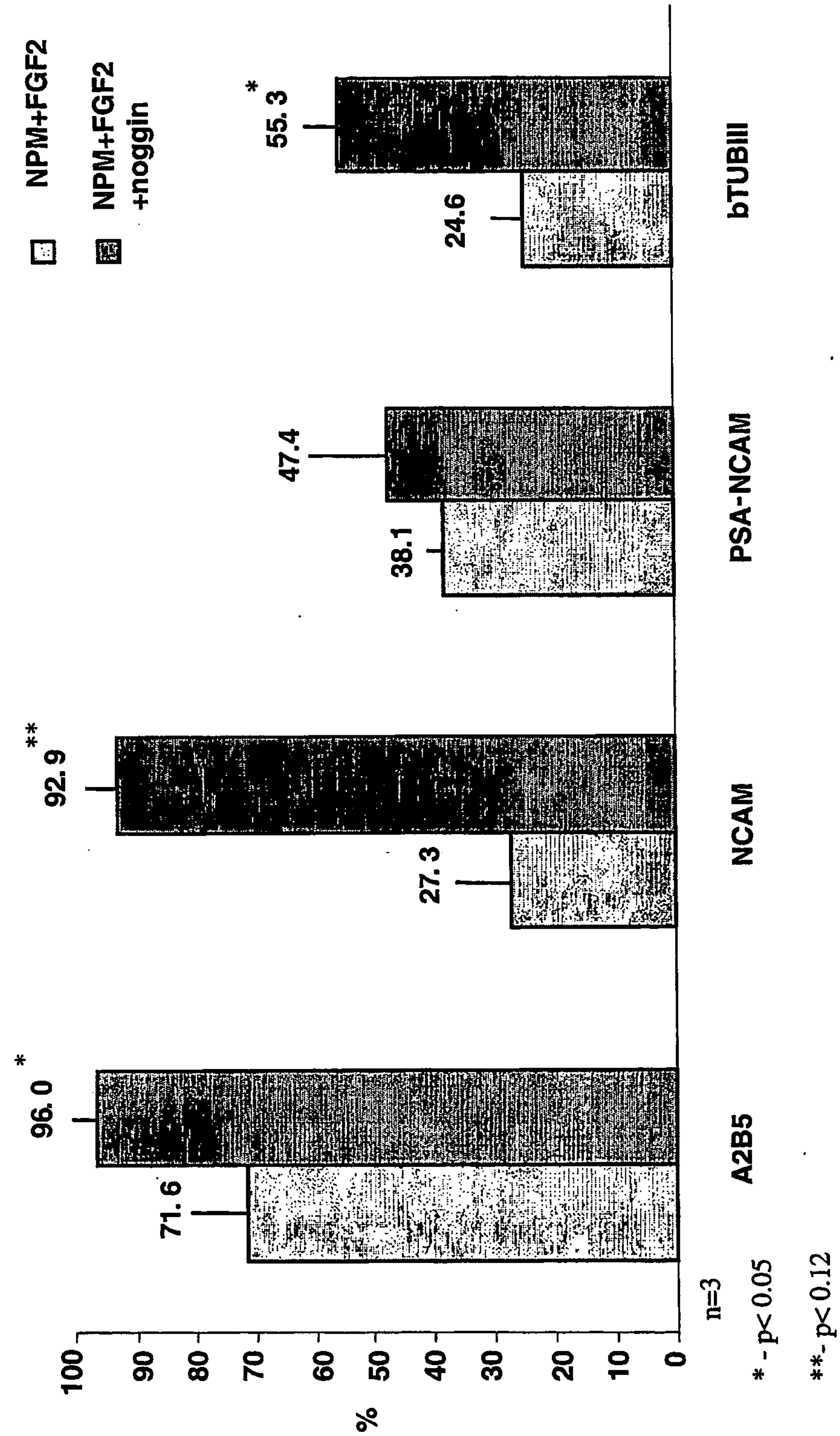
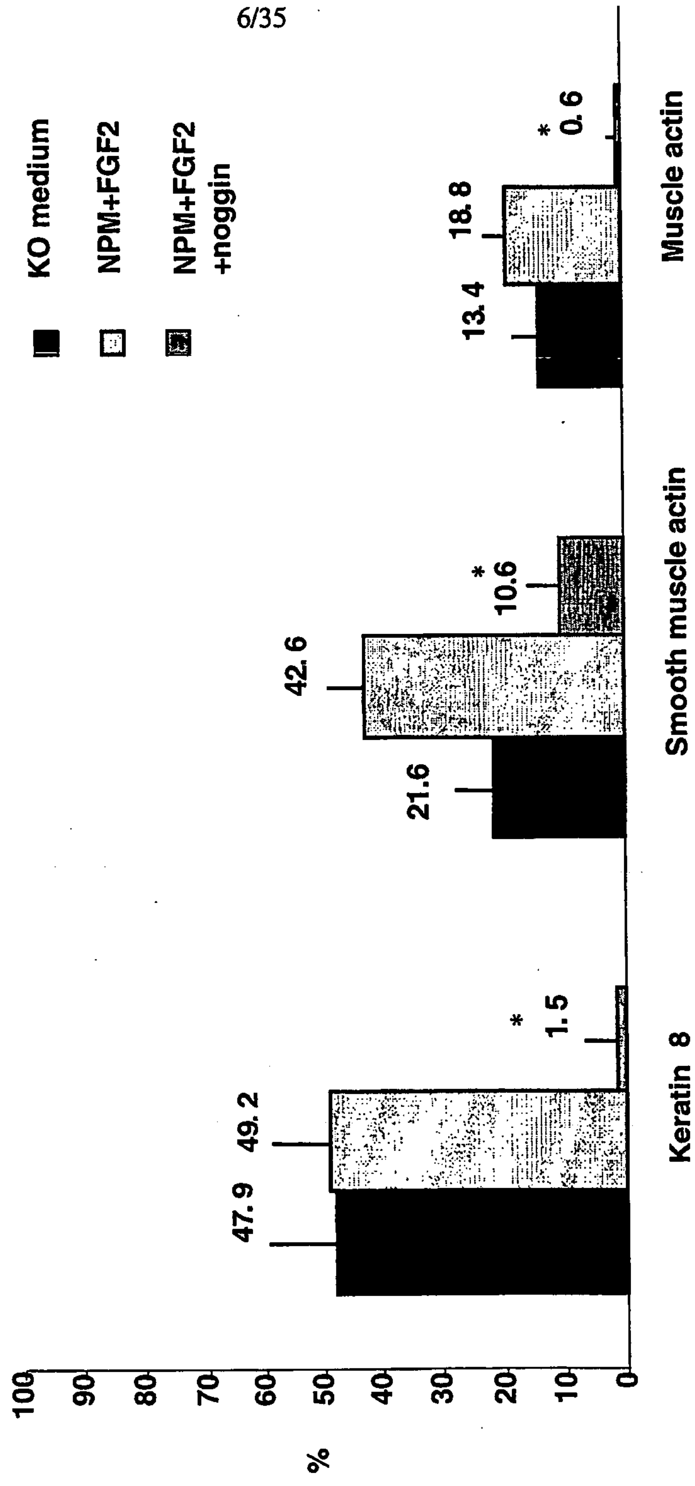

NPM+FGF2
NPM+FGF2 +noggin
%
100
90
80
70
60
50
40
30
20
10
0
71.6
96.0 *
27.3
92.9 **
38.1
47.4
24.6
55.3 *
A2B5
NCAM
PSA-NCAM
bTUBIII
n=3
* - p< 0.05
**- p< 0.12

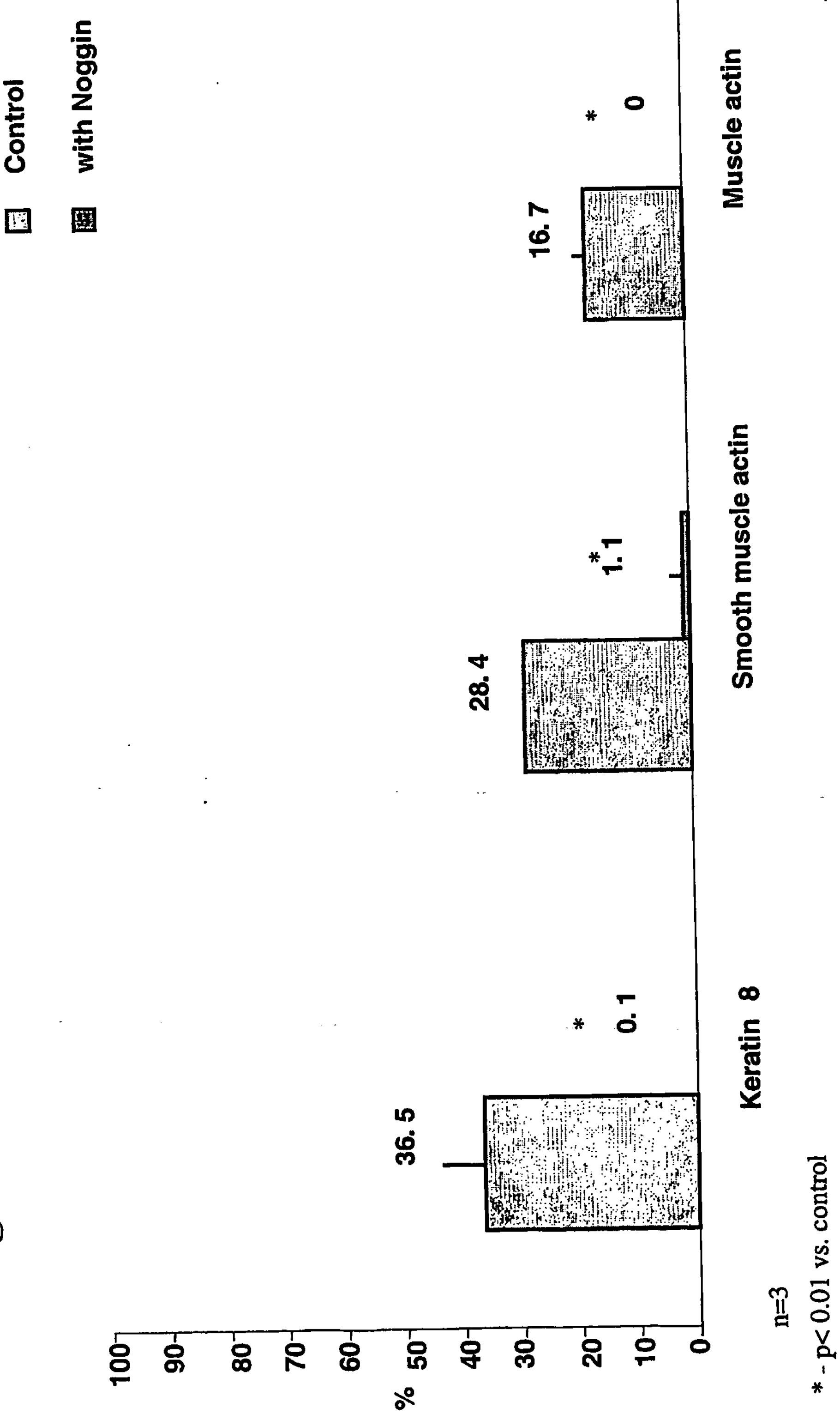
Fig 8




% represents the number of stained cells with a specific antibody from the total of 200 cells, within 4 independent experiments.

* p< 0.01 vs. NPM+FGF2

Fig 9


Control
with Noggin
%
100
90
80
70
60
50
40
30
20
10
0
36.5
* 0.1
Keratin 8
28.4
* 1.1
Smooth muscle actin
16.7
* 0
Muscle actin
n=3
* - p< 0.01 vs. control

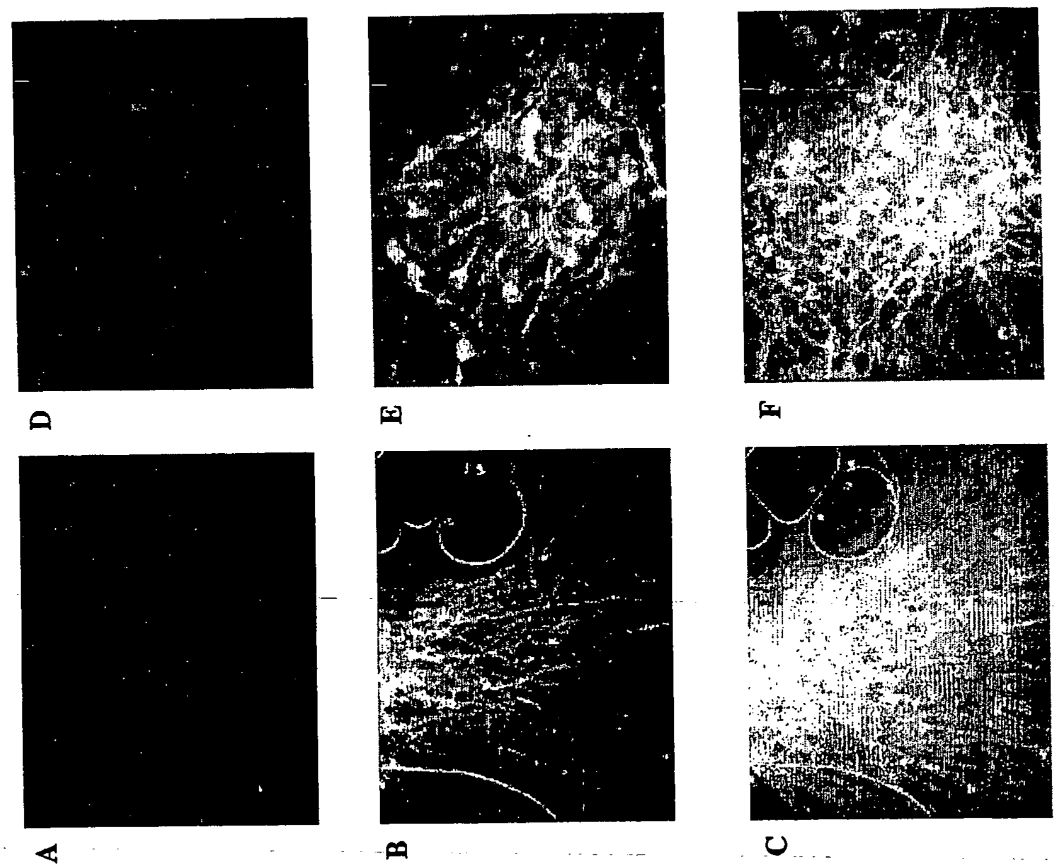



Fig 10

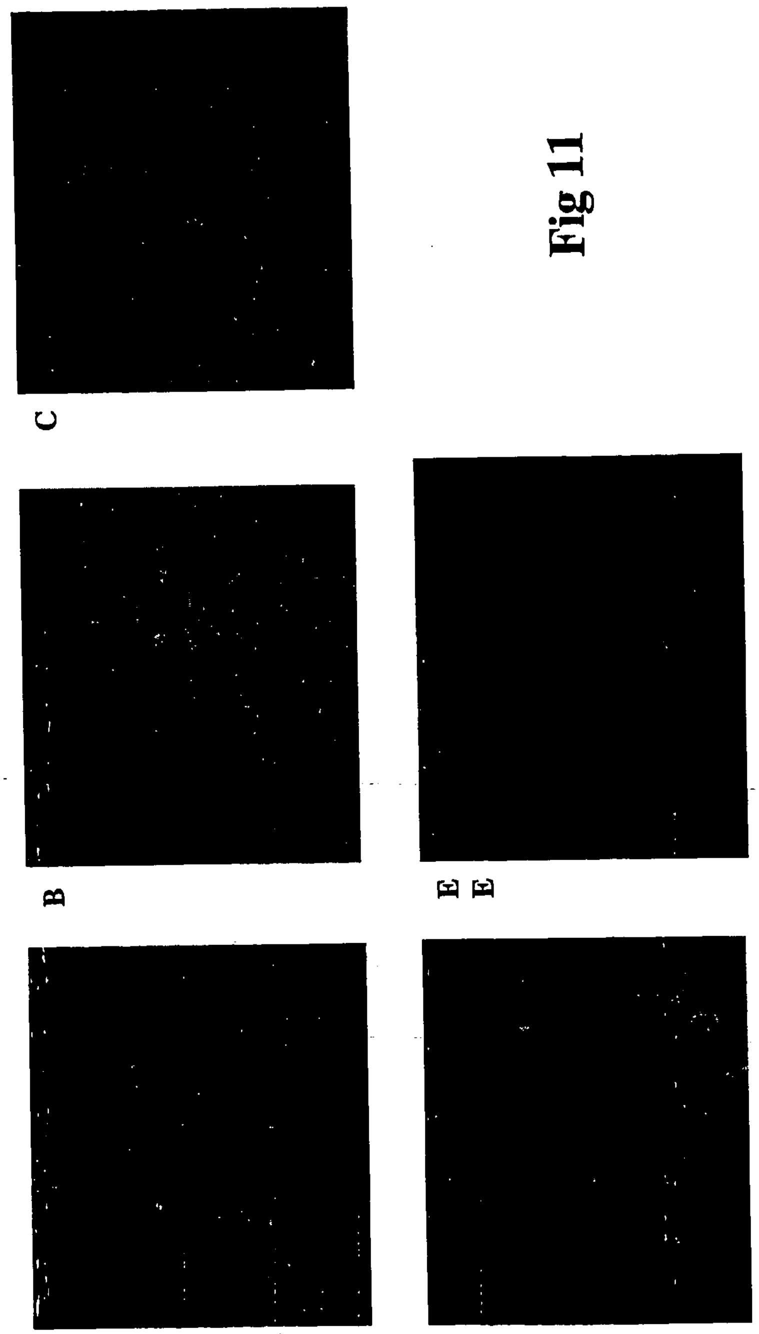
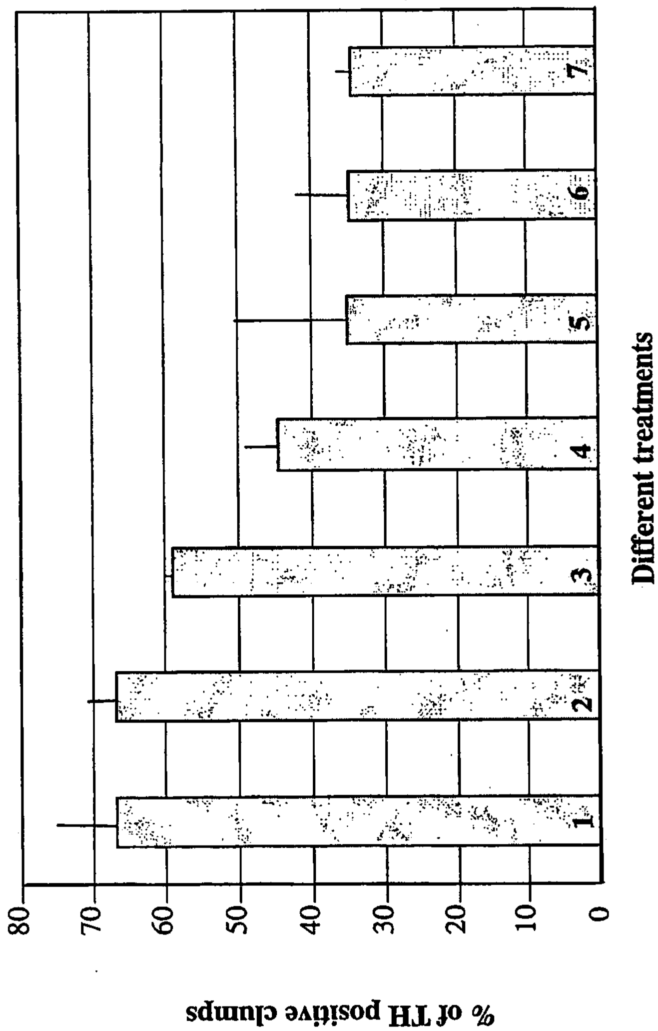
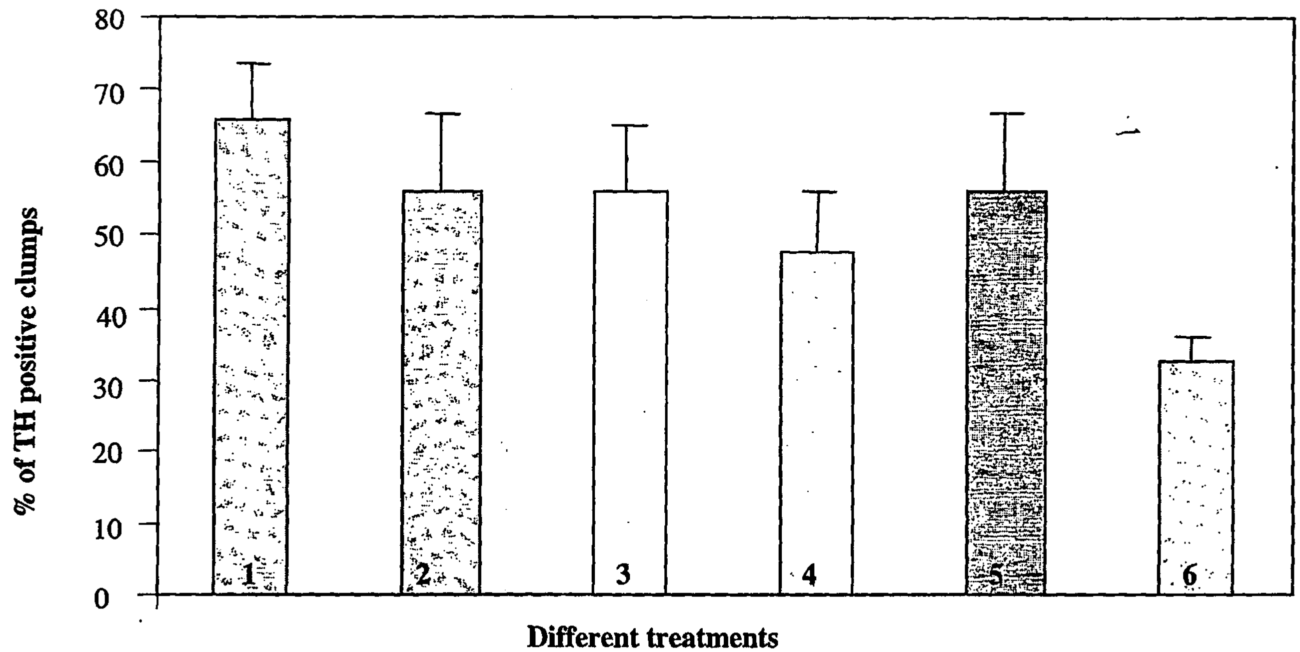



Fig 11




1 FGF8 (200ng/ml) &SHH (1µg/ml)& ascorbic acid (AA) ( 800µM/ml)
2 FGF8 (200ng/ml)&AA (800µM/ml)
3 FGF17 (200ng/ml)&AA (800µM/ml)
4 FGF17 (100ng/ml)& AA (400µM/ml)
5 FGF8 (100ng/ml)& FGF17 (100ng/ml)& AA(400µM/ml)
6 AA (800µM/ml)
7 SHH (1µg/ml)&AA (800µM/ml)

**Fig 12**




1 FGF1 (200ng/ml), IBMX (0.25mM), forskolin (50μM), TPA (200nM), dopamine (20μM), ascorbic acid(AA) (400μM)
2 FGF1 (100ng/ml), IBMX (0.25mM), forskolin (50μM), TPA (200nM), dopamine (20μM), AA (400μM)
3 FGF8 (100ng/ml), IBMX (0.25mM), forskolin (50μM), TPA (200nM), dopamine (20μM), AA (400μM)
4 FGF8 (200ng/ml), IBMX (0.25mM), forskolin (50μM), TPA (200nM), dopamine (20μM) AA (400μM)
5 IBMX ( 0.25mM), forskolin (50μM), TPA (200nM), dopamine(20μM), AA (400μM)
6 Control DMSO, AA (400μM)

Fig 13

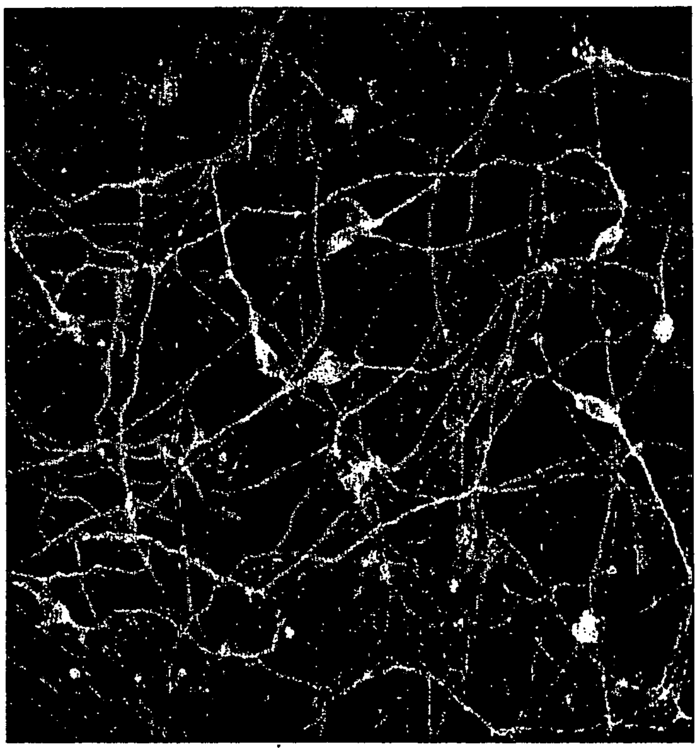


Fig 14

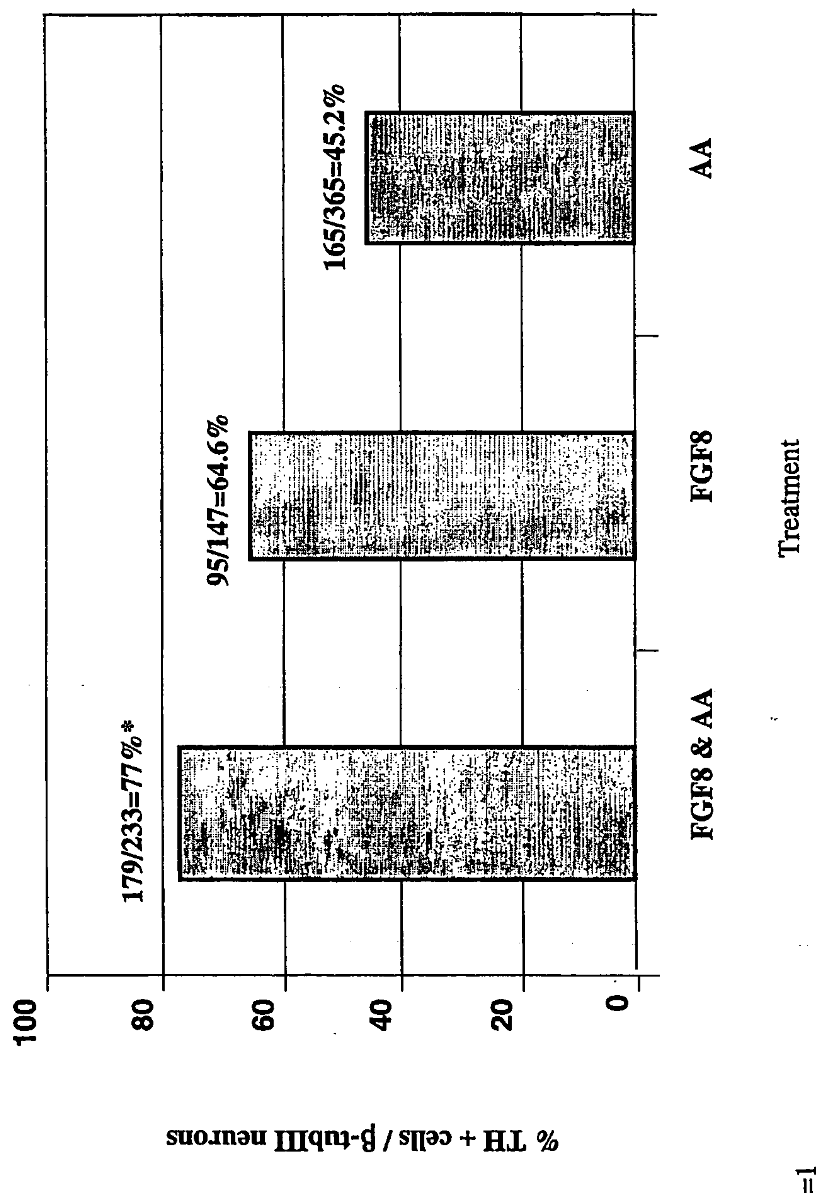



Fig 15

n=1

% Represents the percentage of TH positive cells from the total number of β-tubulinIII positive cells.

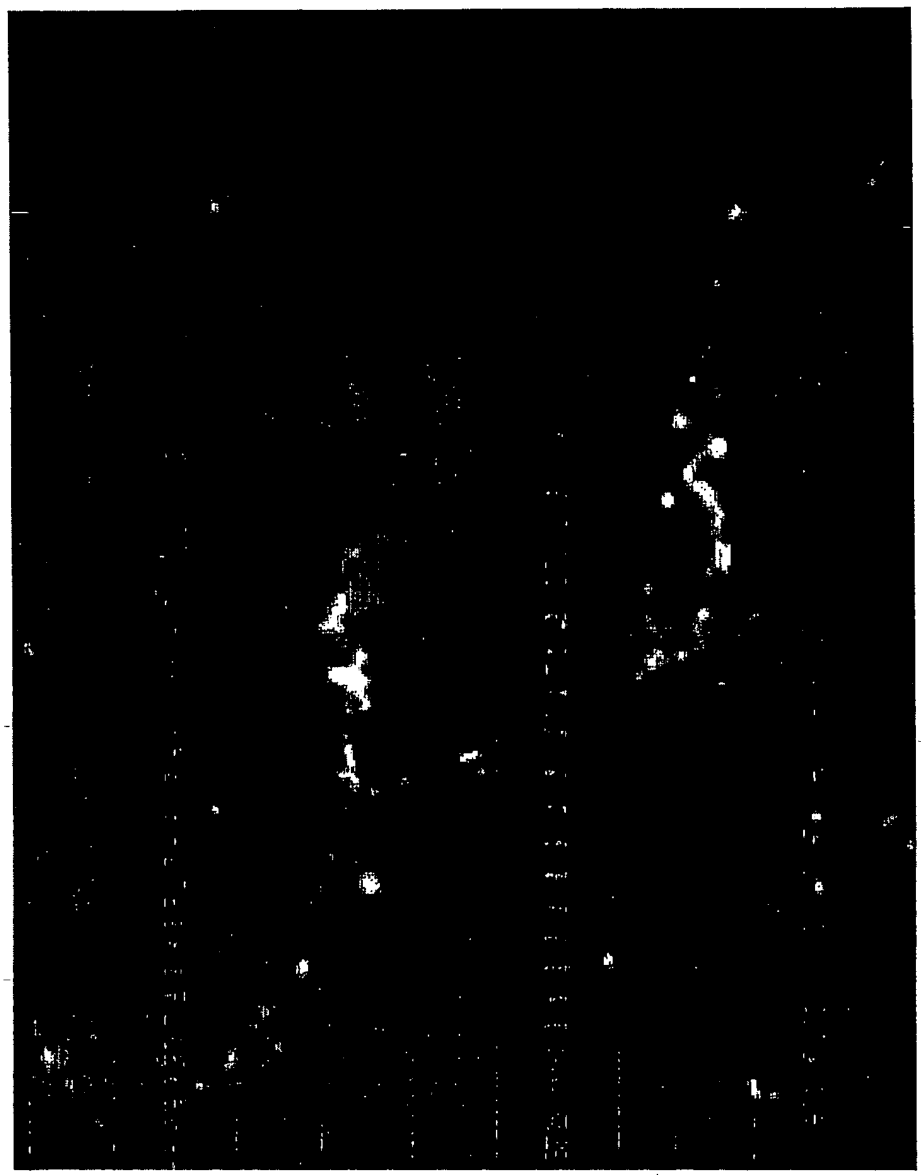
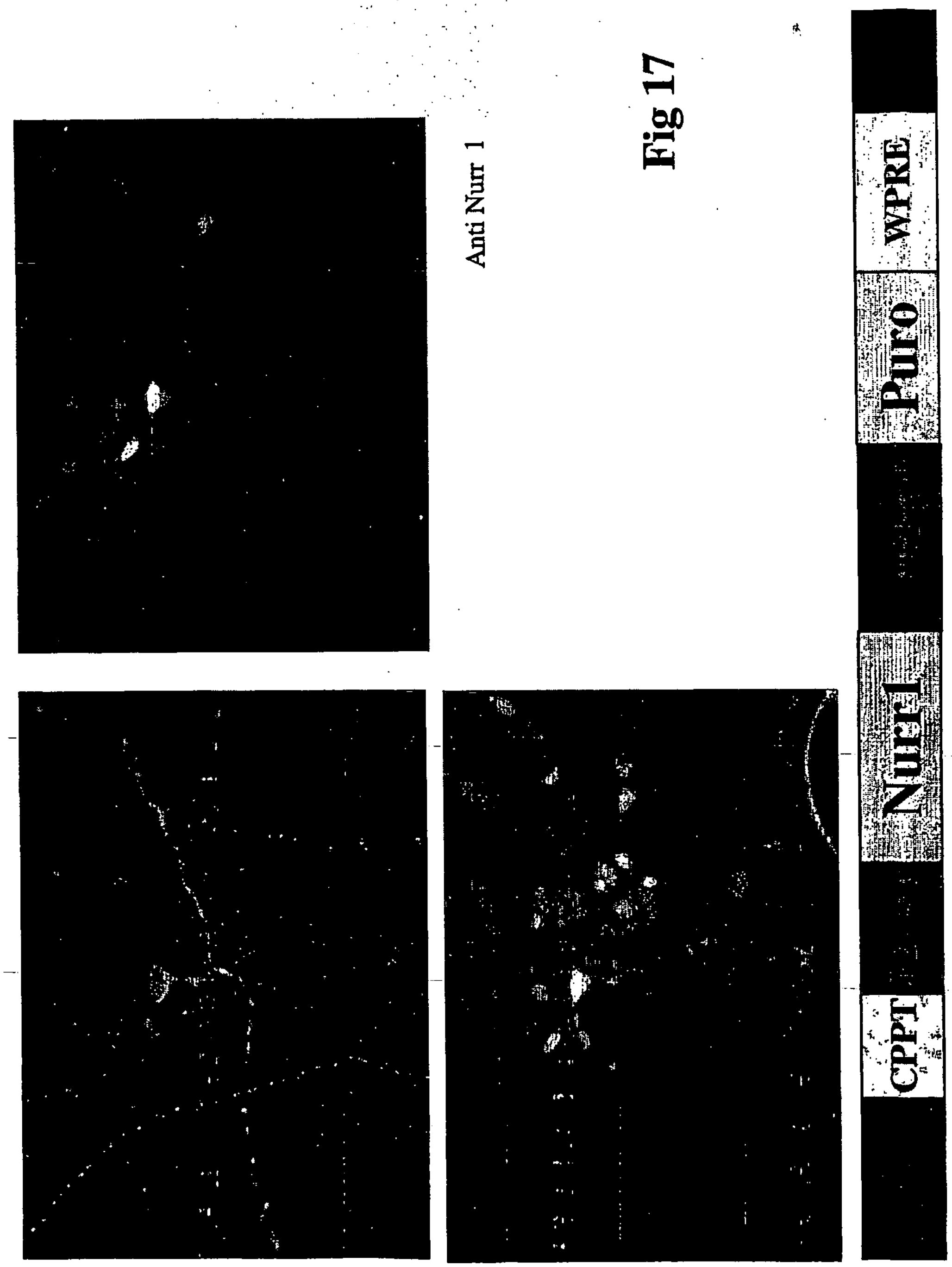


Fig 16

Fig 17

A



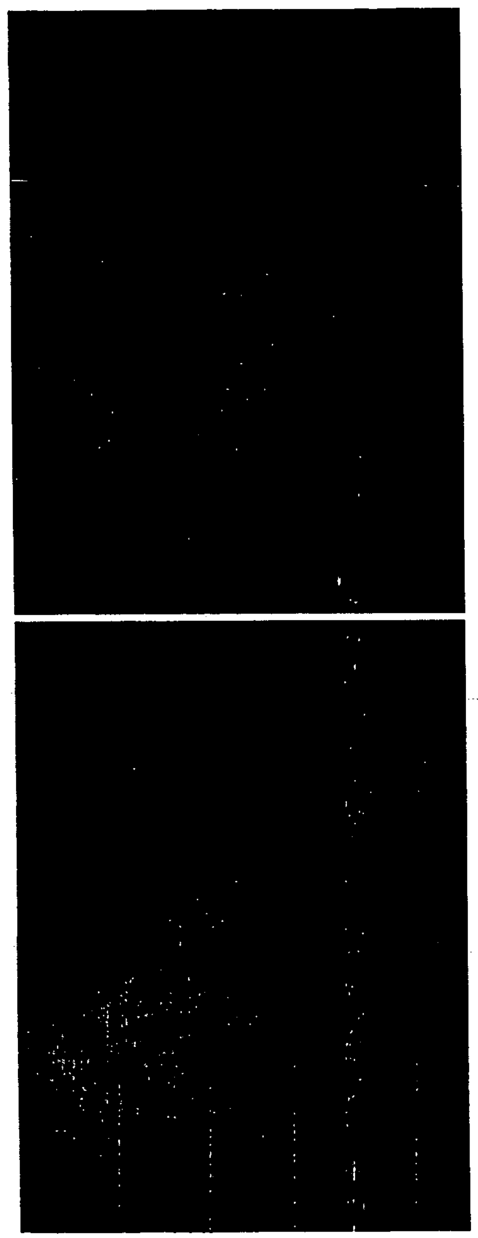

Anti Nurr 1

Anti TH

Anti TH &Nurr1

B

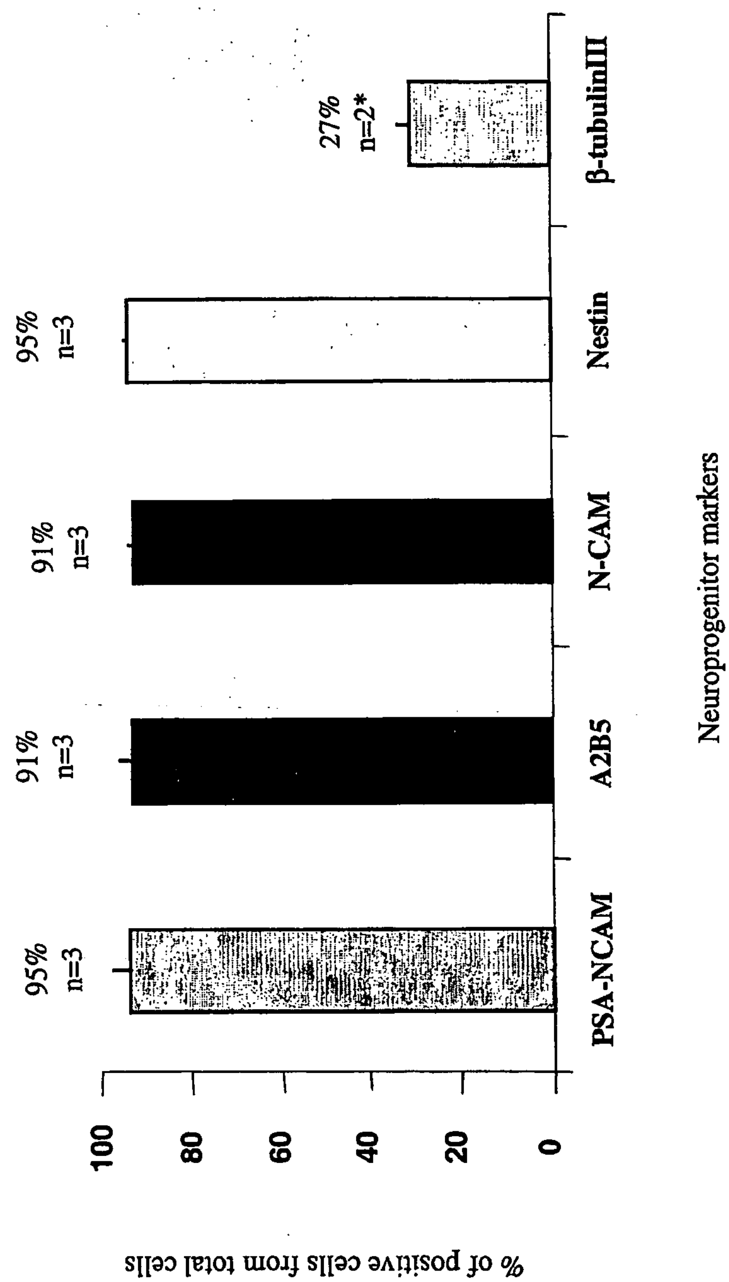
Fig 18



Lesioned side

Intact side

Fig 19



95%
n=3
91%
n=3
91%
n=3
95%
n=3
27%
n=2*
100
80
60
40
20
0
PSA-NCAM
A2B5
N-CAM
Nestin
β-tubulinIII
Neuroprogenitor markers
% of positive cells from total cells

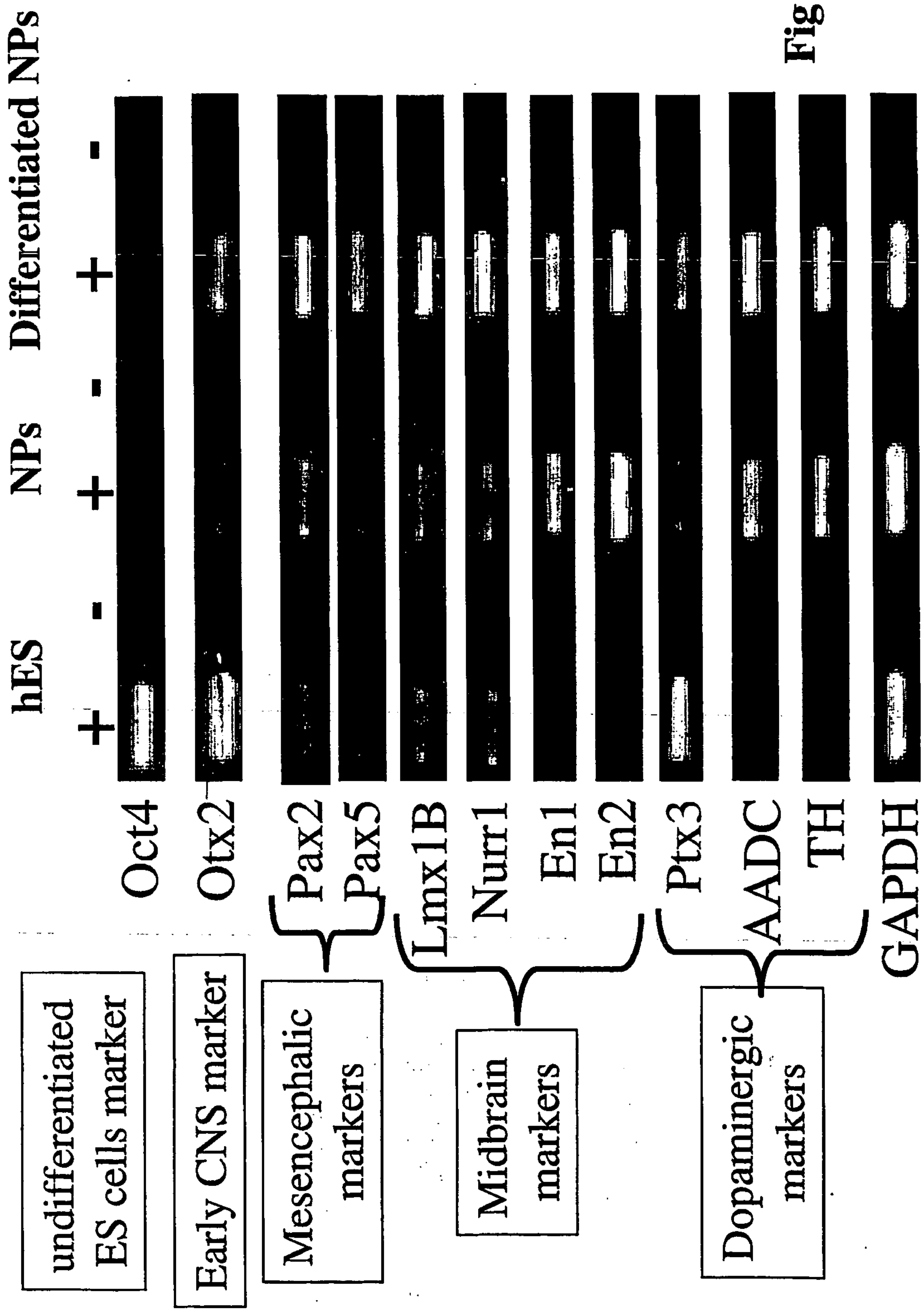

hES
NPs
Differentiated NPs
+
-
+
-
+
-
Oct4
Otx2
Pax2
Pax5
Lmx1B
Nurr1
En1
En2
Ptx3
AADC
TH
GAPDH
undifferentiated ES cells marker
Early CNS marker
Mesencephalic markers
Midbrain markers
Dopaminergic markers

Fig 20

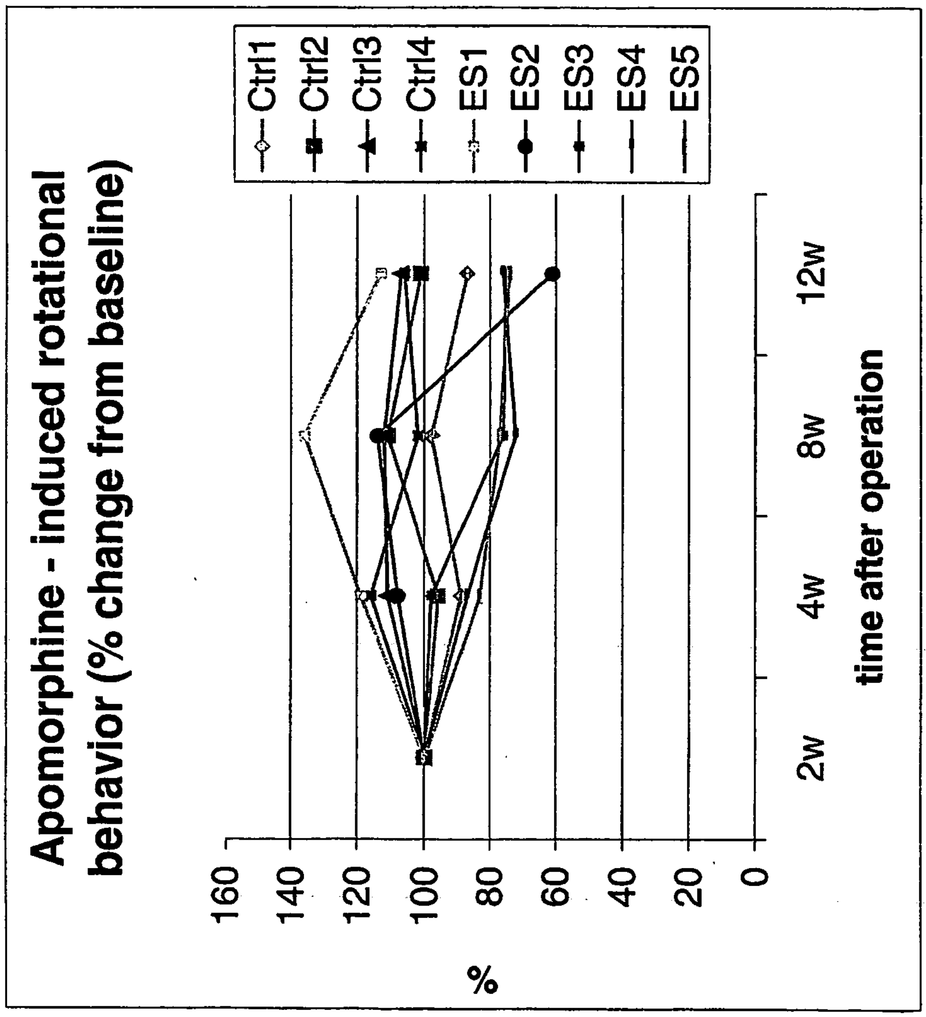

Apomorphine - induced rotational behavior (% change from baseline)
%
160
140
120
100
80
60
40
20
0
2w
4w
8w
12w
time after operation
Ctrl1
Ctrl2
Ctrl3
Ctrl4
ES1
ES2
ES3
ES4
ES5

Fig 21

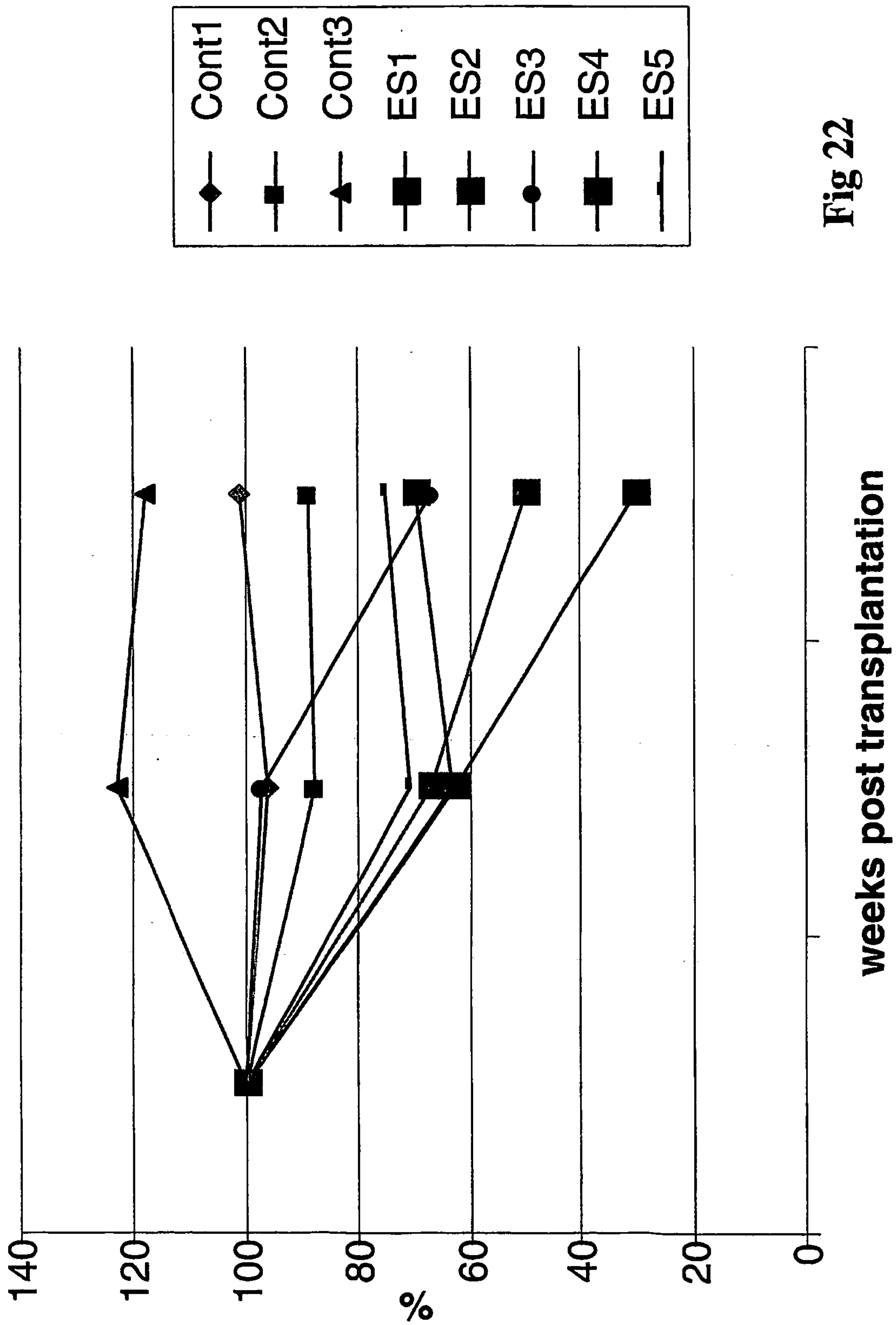
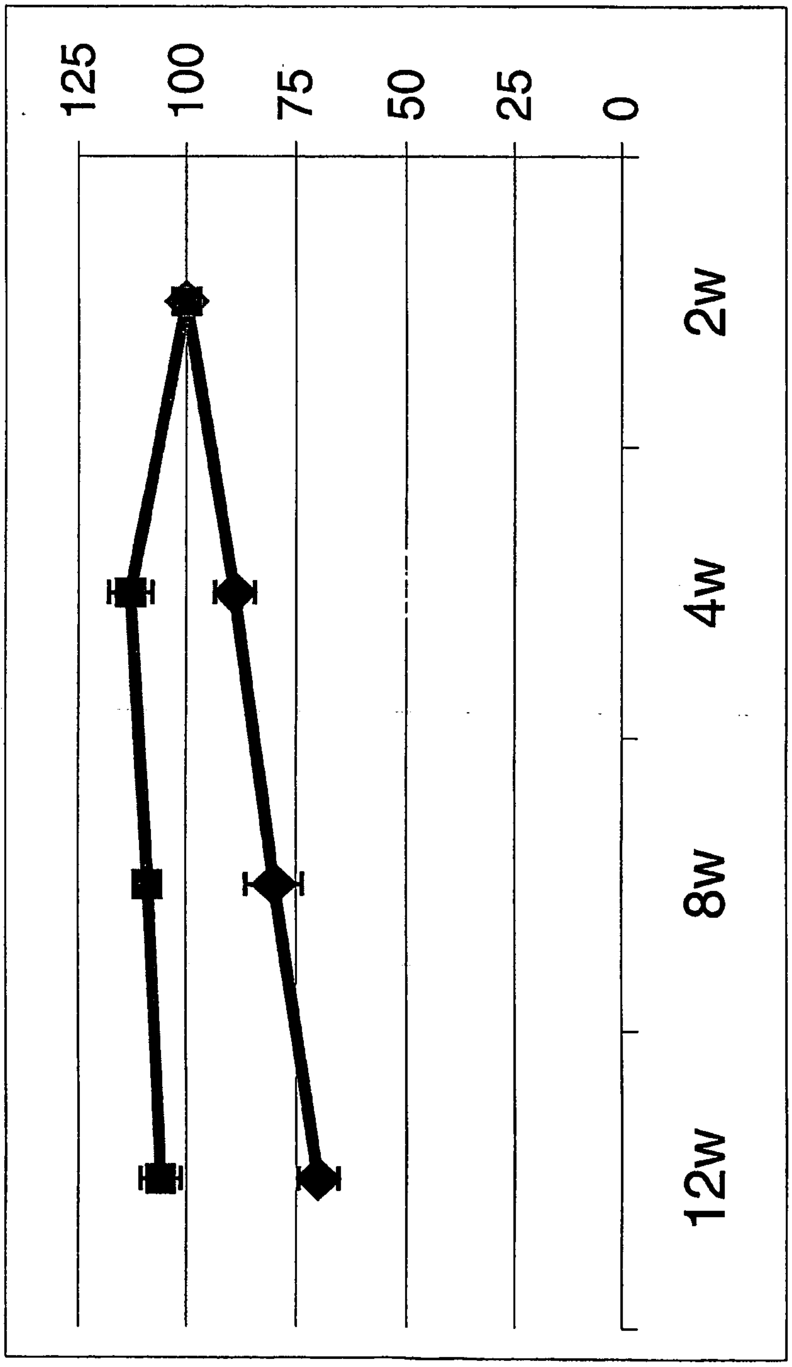

Cont1
Cont2
Cont3
ES1
ES2
ES3
ES4
ES5
140
120
100
80
60
40
20
0
%
weeks post transplantation

Fig 22

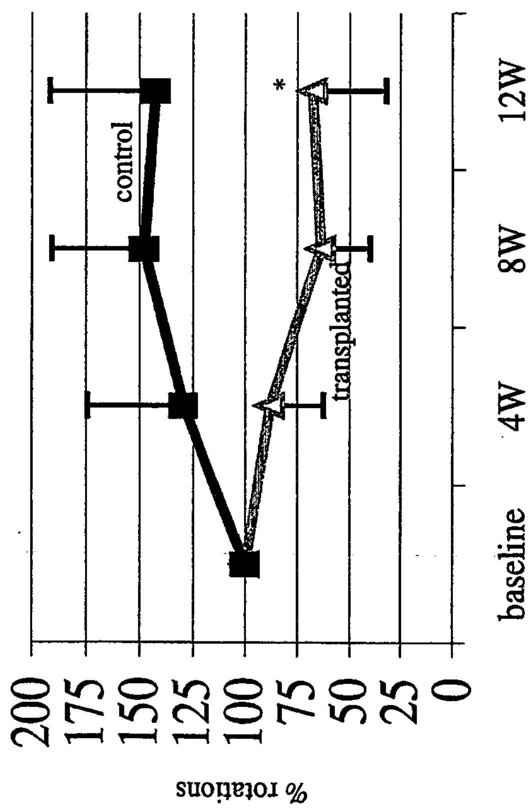
Fig 23




**HNS: n=16**

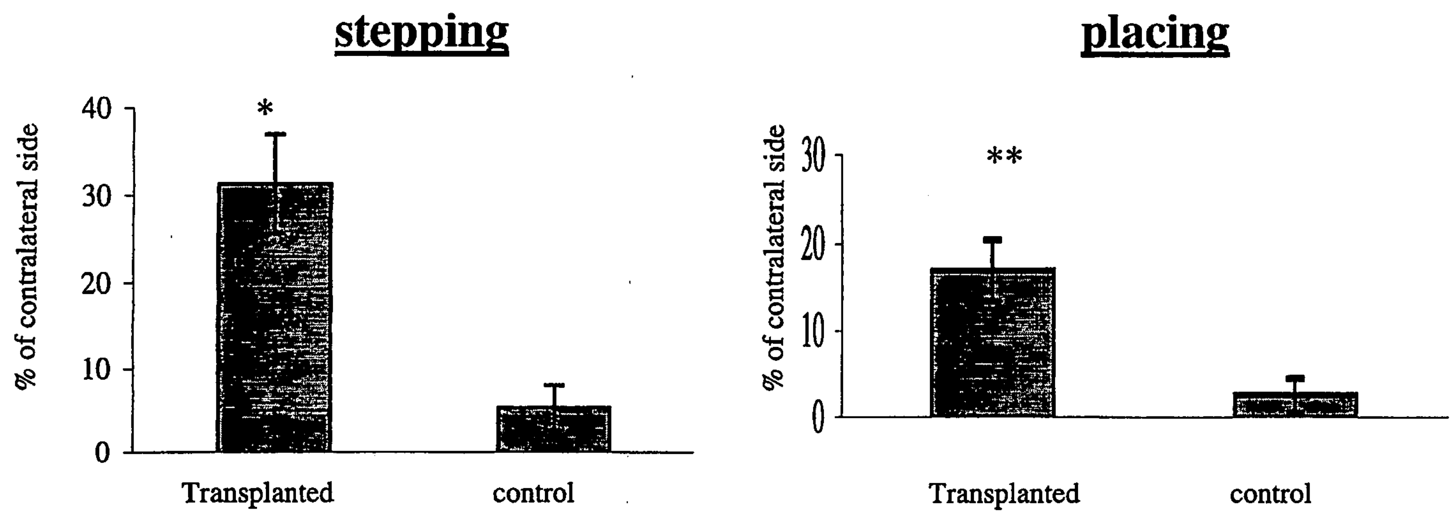
**Control: n=12**

*p<0.05 as compared to 2weeks and to the corresponding control group




Transplanted, n=11; control, n=10
* p=0.00037 as compared to control animals at 12 weeks
p=0.0036 as compared to baseline rotations at 2 weeks

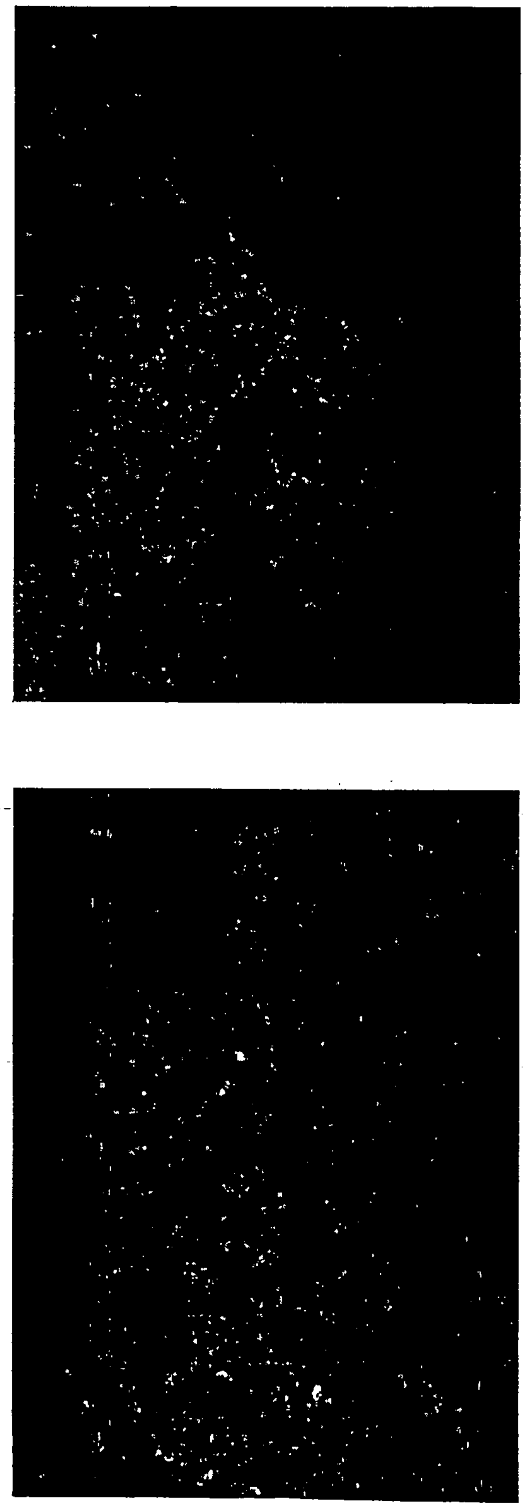
**Fig 24**




Transplanted, n=11, mean = 17+/-3.5;
Control, n=10, mean = 2.65+/-2
* P=0.0026 as compared to control

Transplanted, n=11, mean = 31.3+/-5.6;
Control, n=10, mean=5.4+/-2.66
** P=0.0007 as compared to control

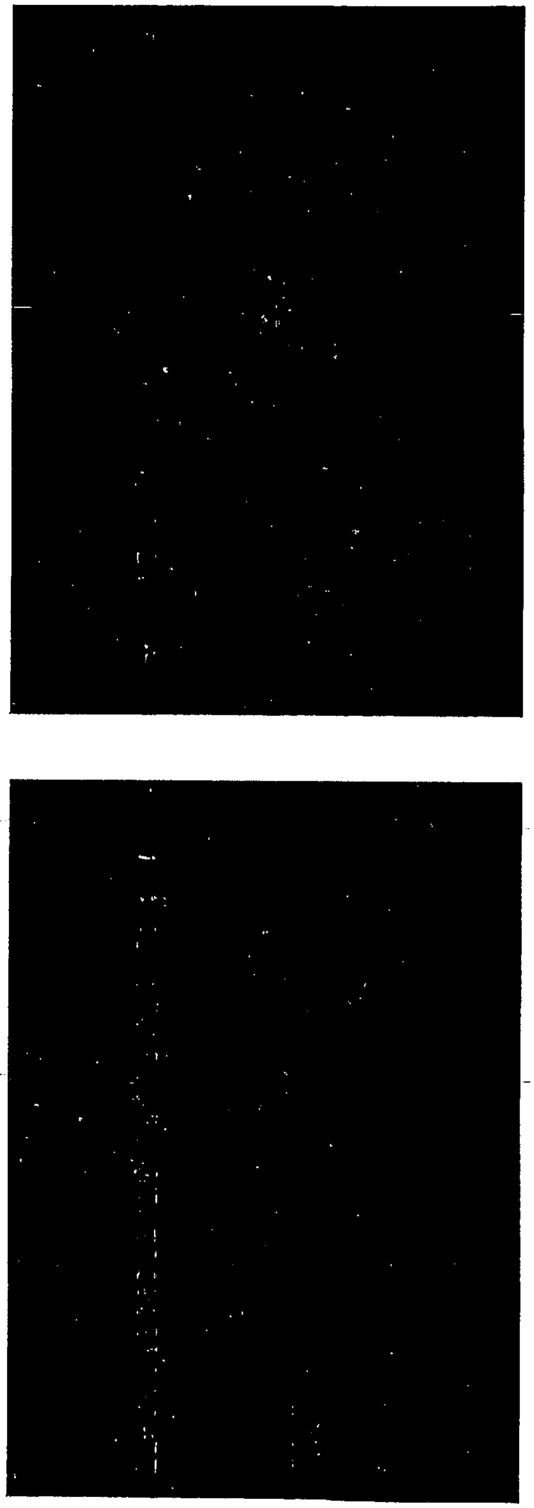
**Fig 25**



B X100

A X100

Fig 26

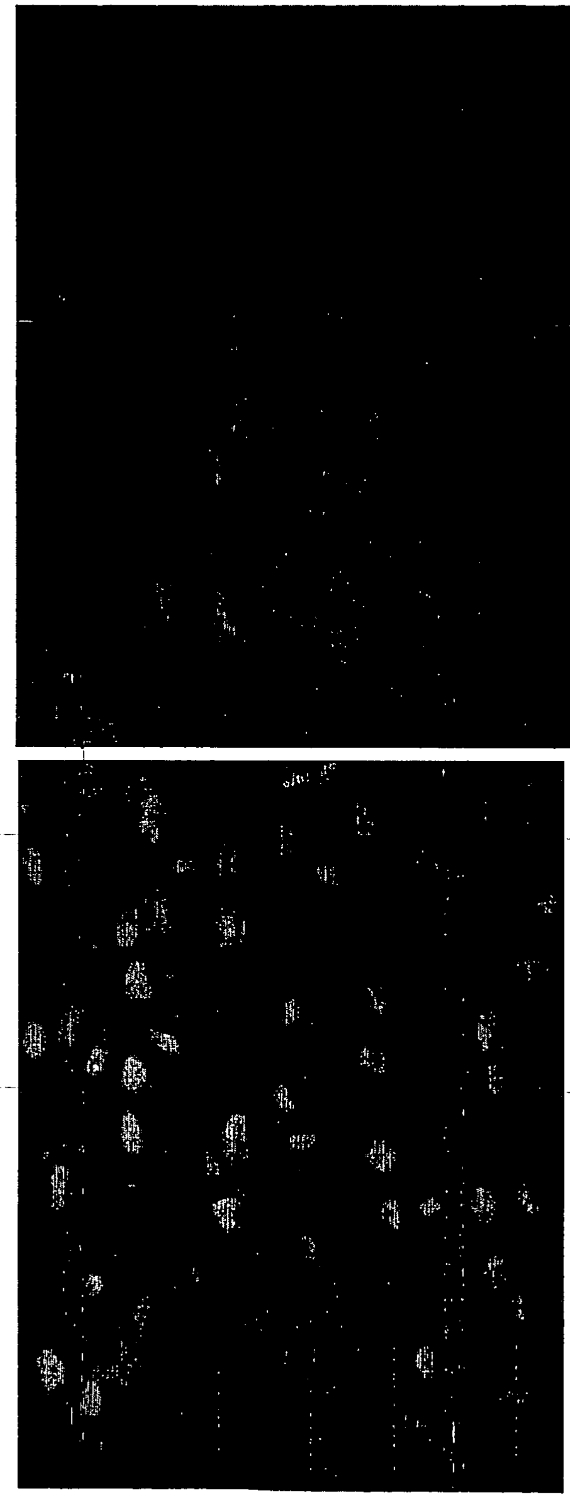


X100

X200

Fig 27

Fig 28



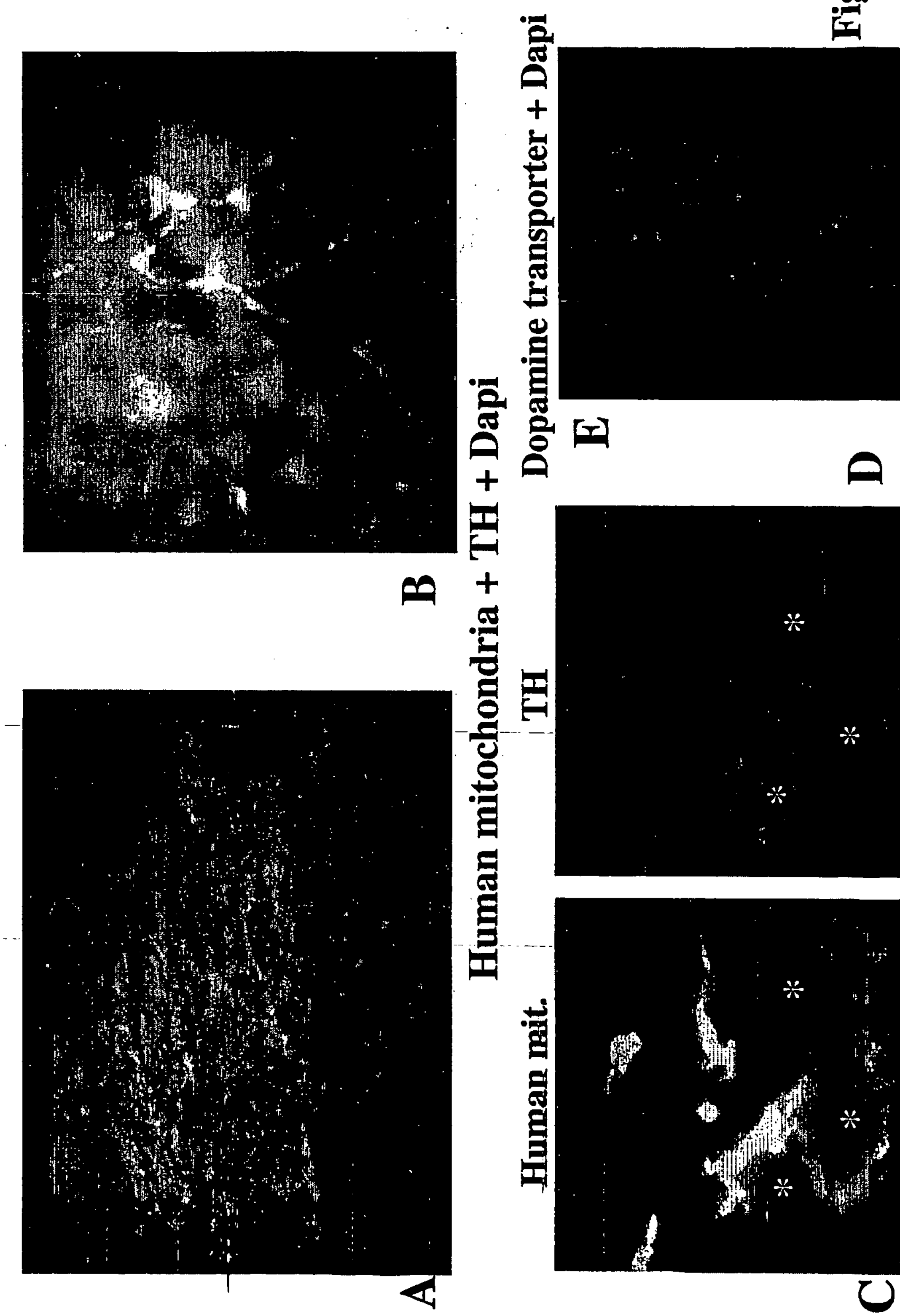
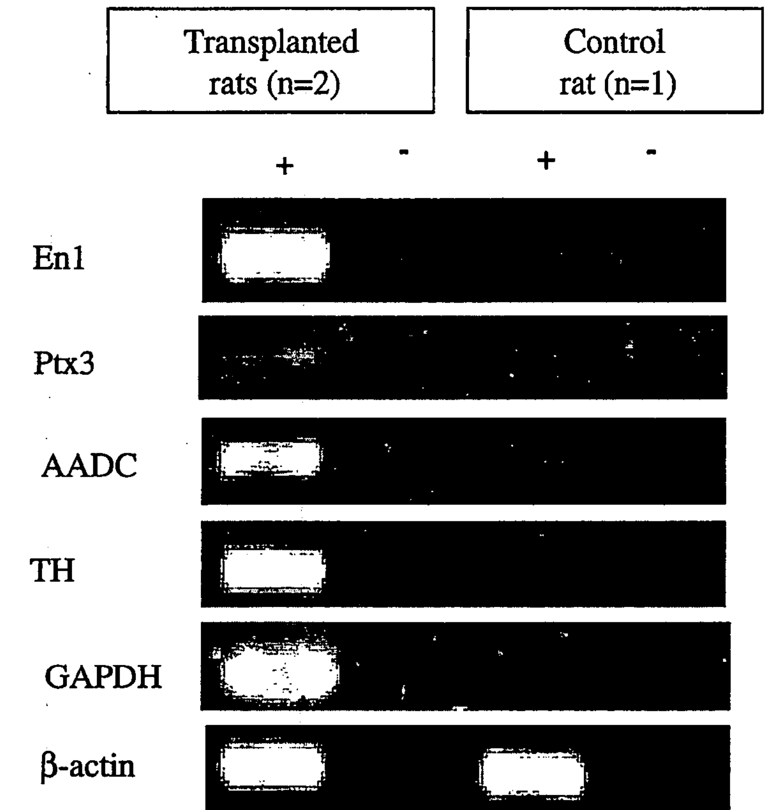

A
B
Human mitochondria + TH + Dapi
C
Human mit.
TH
D
E
Dopamine transporter + Dapi

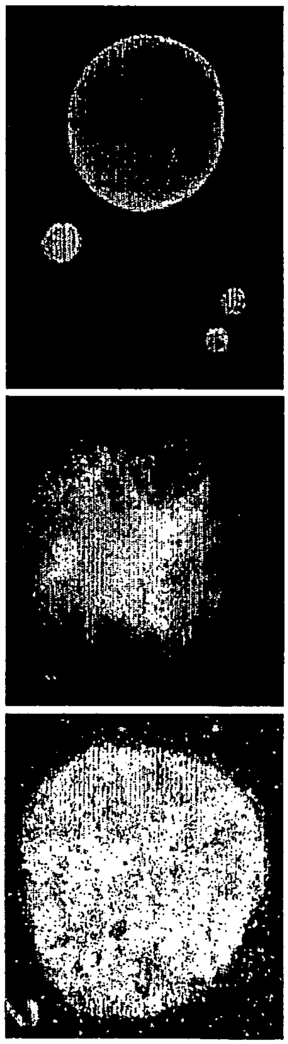
Fig 29




Human specific primers were designed for the amplification of AADC, *En1*, TH, *Ptx3*, GAPDH

The RT-PCR primers for β-actin were not human specific

**Fig 30**



A B C

Fig 31

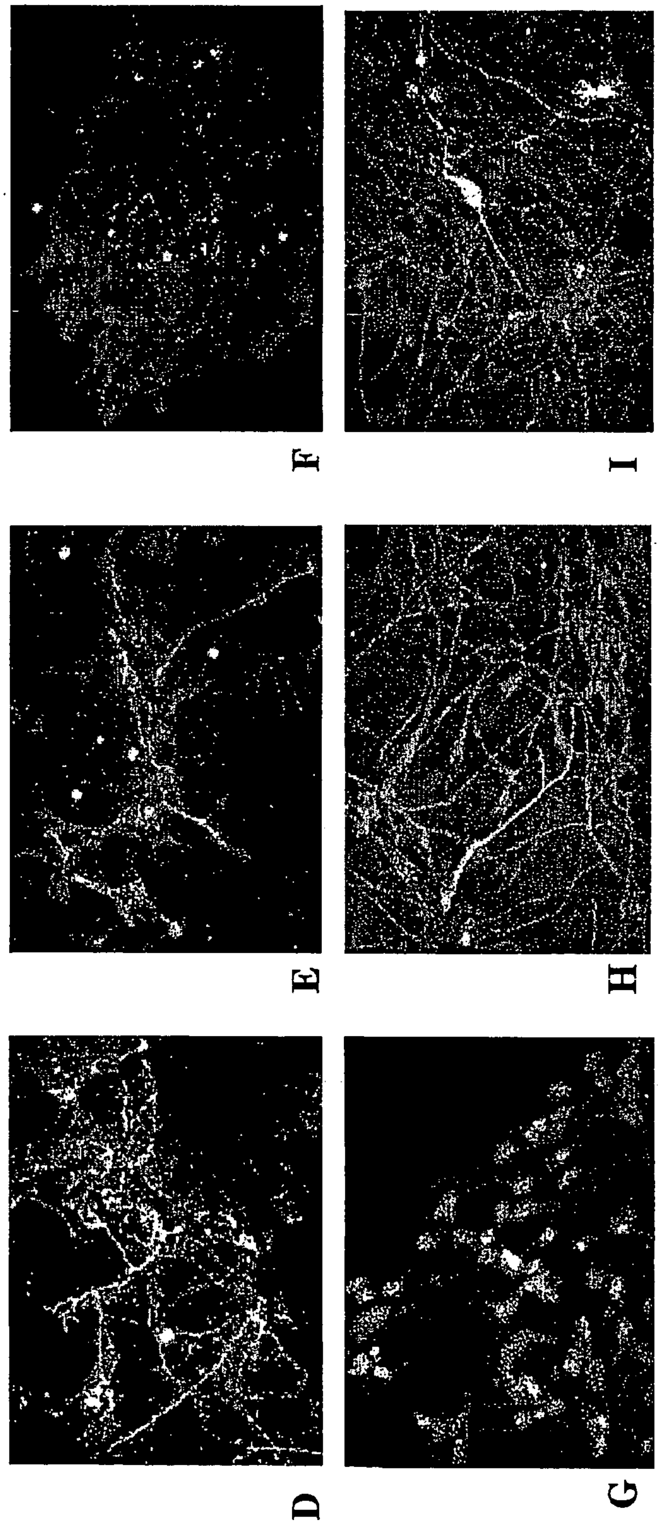



Fig 31

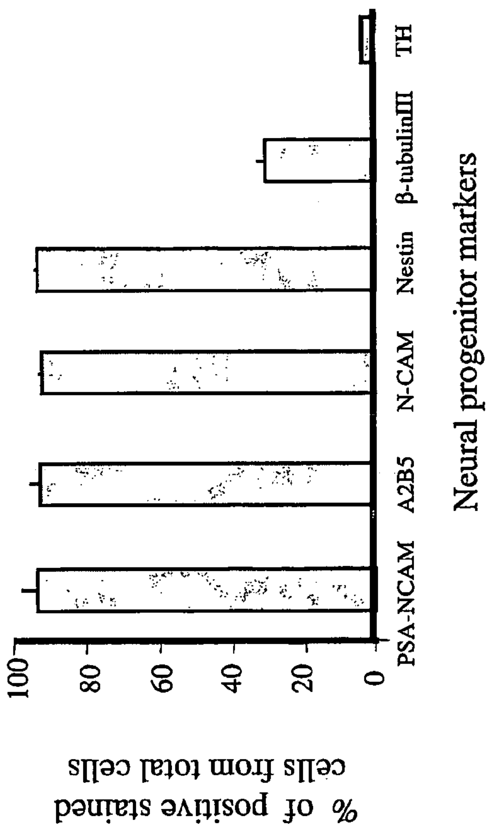

% of positive stained cells from total cells
100
80
60
40
20
0
PSA-NCAM
A2B5
N-CAM
Nestin
β-tubulinIII
TH
Neural progenitor markers

Fig 31

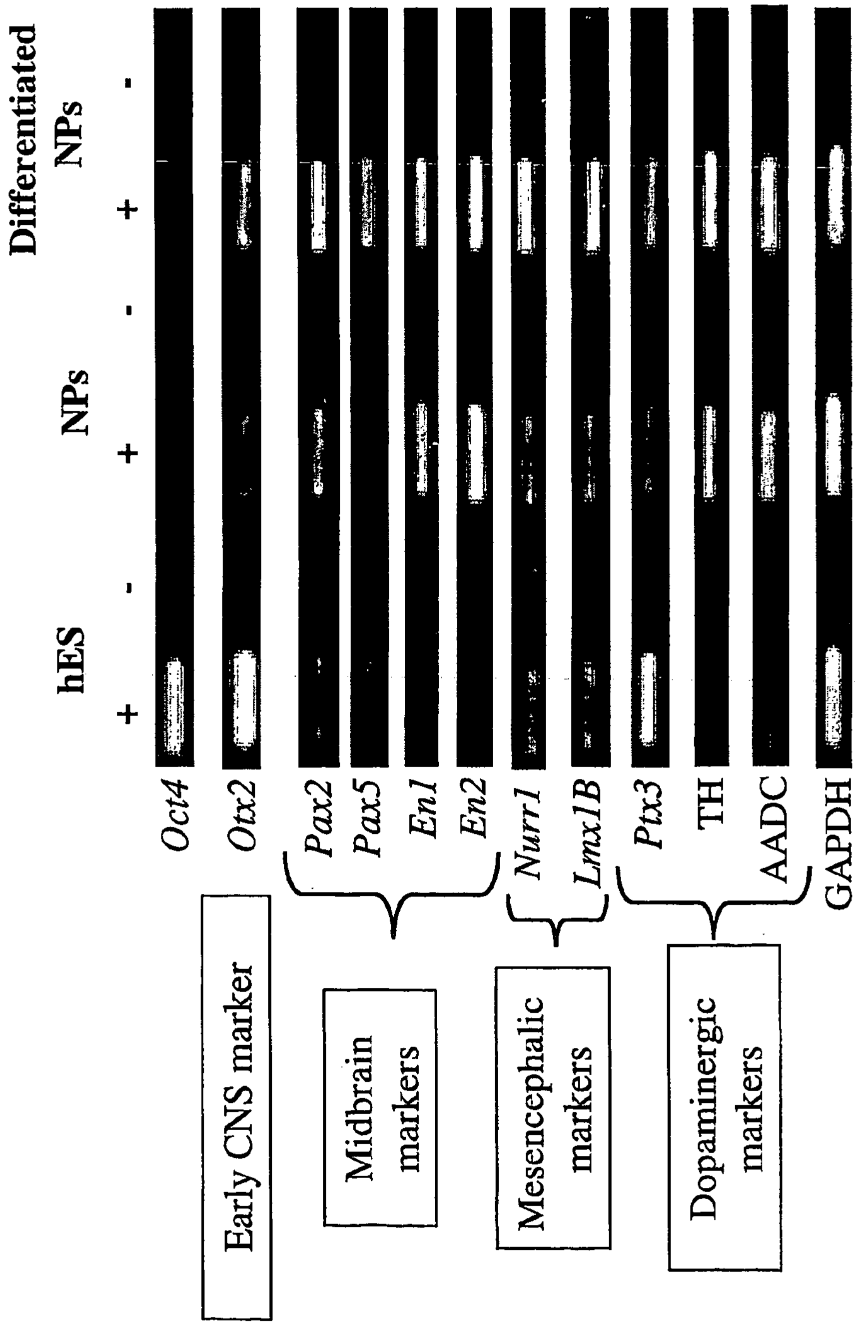
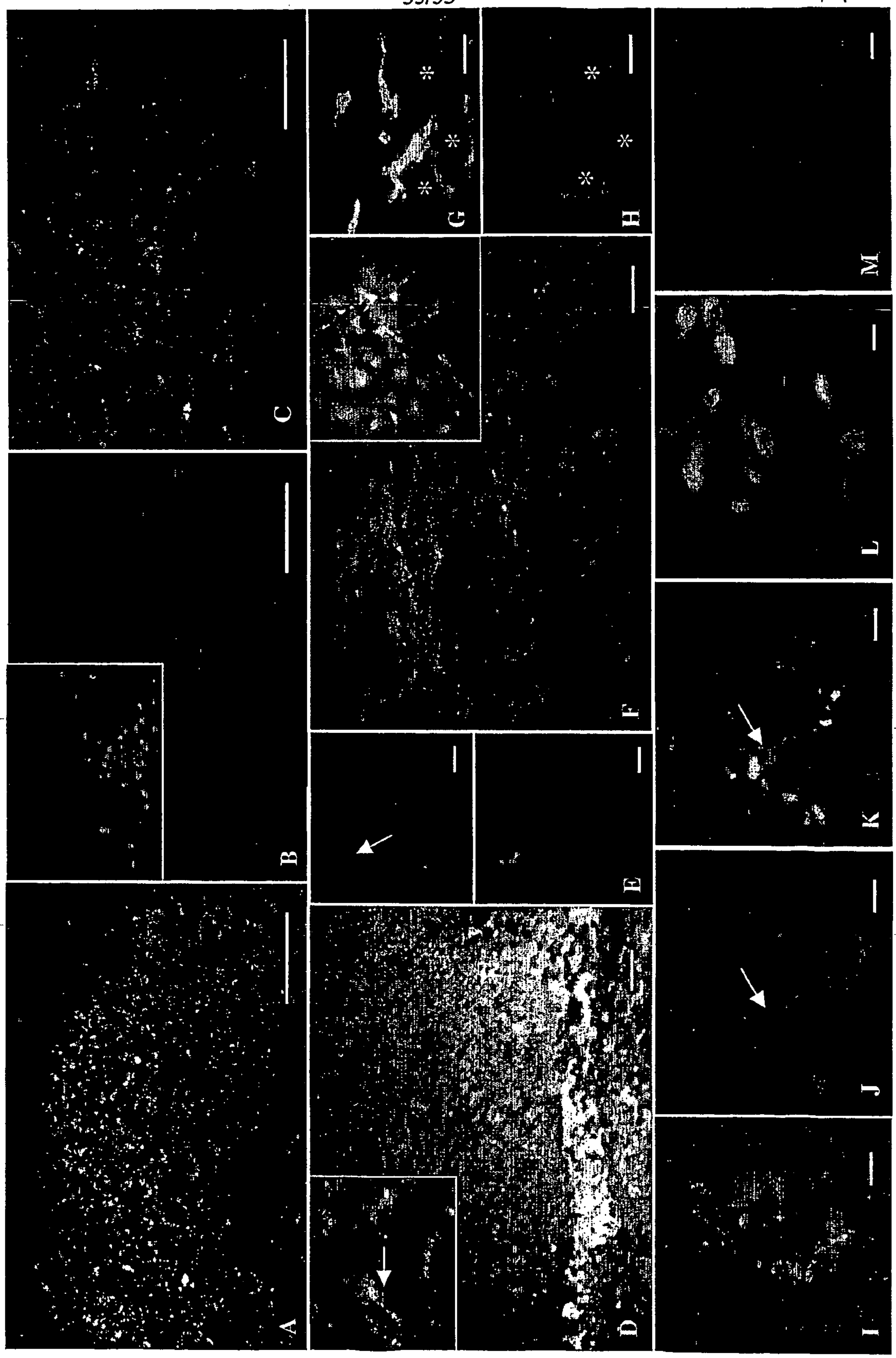

hES
NPs
Differentiated NPs
+
-
+
-
+
-
Oct4
Otx2
Pax2
Pax5
En1
En2
Nurr1
Lmx1B
Ptx3
TH
AADC
GAPDH
Early CNS marker
Midbrain markers
Mesencephalic markers
Dopaminergic markers

Fig 32

Fig 33



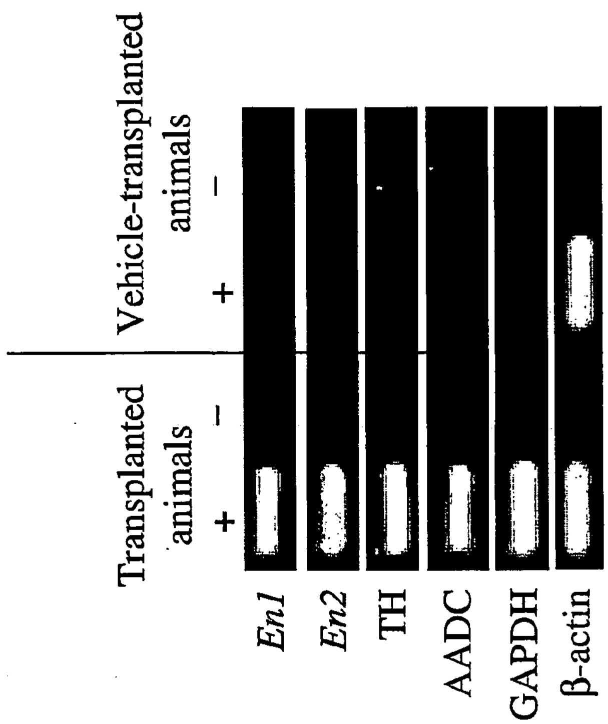

Transplanted animals
Vehicle-transplanted animals
+
–
+
–
En1
En2
TH
AADC
GAPDH
β-actin

Fig 34

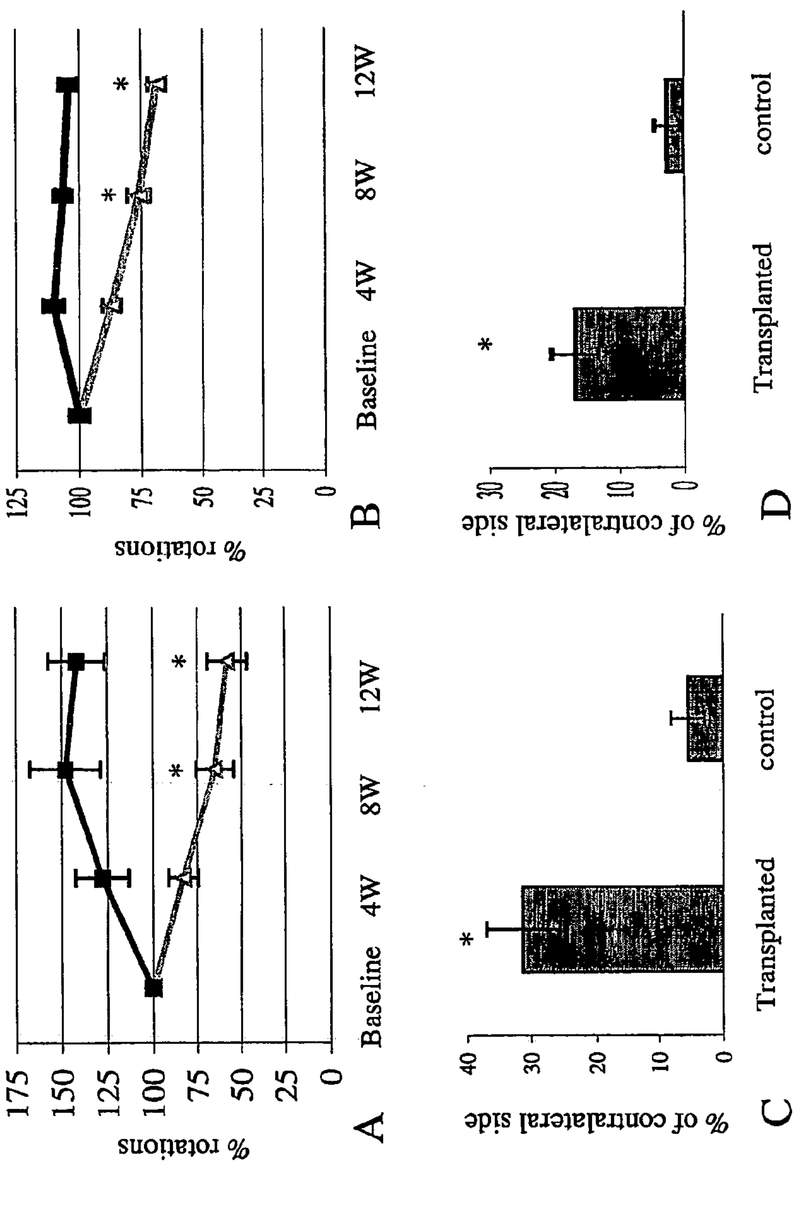

A
% rotations
175
150
125
100
75
50
25
0
Baseline
4W
8W
12W
*
*
B
% rotations
125
100
75
50
25
0
Baseline
4W
8W
12W
*
*
C
% of contralateral side
40
30
20
10
0
Transplanted
control
*
D
% of contralateral side
30
20
10
0
Transplanted
control
*

Fig 35

| INTERNATIONAL SEARCH REPORT | International application No. PCT/AU03/00704 |
|---|---|

**A. CLASSIFICATION OF SUBJECT MATTER**

Int. Cl. [7]: C12N 5/08

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA, WPIDS, MEDLINE: Keywords - embryonic stem cell, ES cell, fibroblast growth factor, FGF, BMP, bone morphogenic protein, fetuin, noggin, chordin, gremlin, follistatin, cerberus, amnionless, DAN, BMRIA

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 02/26941, A (Van der Kooy D & Tropepe V) 4 April 2002. See whole document. | 1-23 & 31-37 |
| X | WO 01/68815, A (Monash University *et al.*) 20 September 2001. See whole document, especially page 34 lines 29-32 and page 38 lines 1-5 and 30-32. | 7-10, 22 & 23 |
| Y | | 1-6, 8-14 & 31-37 |

[X] Further documents are listed in the continuation of Box C   [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 August 2003 | - 6 AUG 2003 |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustralia.gov.au<br>Facsimile No. (02) 6285 3929 | Authorized officer<br>**JANE MCHENRY**<br>Telephone No : (02) 6283 2091 |

**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages *(Remove spaces when completed if the page is too long)* | Relevant to claim No. |
|---|---|---|
| X | WO 00/27995, A (Monash University *et al.*) 18 May 2000. See whole document. | 7-23 |
| X | WO 01/98463, A (Monash University *et al.*) 27 December 2001. See whole document. | 7, 11-14, 22 & 23 |
| Y | | 1-6, 8-10 & 31-37 |
| X | Reubinoff B E *et al.* (2000) Nature Biotechnol. 18(4): 399-404. "Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro" See whole document. | 7, 22 & 23 |
| X | Lillien L & Raphael H (2000) Development 127: 4993-5005. "BMP and FGF regulate the development of EGF-responsive neural progenitor cells" See whole document. | 1-23 & 31-37 |
| X | Schuldiner M *et al.* (2000) Proc. Natl. Acad. Sci USA 97 (21): 11307-11312. "Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells" See whole document. | 7, 22 & 23 |
| P, X | WO 02/081663, A (Japan Science and Technology Corporation) 17 October 2002. See whole document. | 7-23 & 31-37 |

**Box I** **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : **7 and 22 and dependent claims 8-10, 19-21 and 23 (in part)**

   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   These are broad claims that are not limited to the technical features of the invention. Hence, these claims do not comply with rule 6.3 of the PCT. Consequently these claims have only been searched with respect to combination of FGF and BMP antagonist being used to direct the human ES cells towards a neural progenitor cell fate.

3. ☐ Claims Nos :

   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II** **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet below.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-23 and 33-37 and claims 31 and 32 (in part)

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

**Supplemental Box**
(To be used when the space in any of Boxes I to VIII is not sufficient)

**Continuation of Box No: II**

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. The fundamental test for unity of invention is specified in Rule 13.2 of the Regulations under the PCT.

"Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred to in Rule 13.1 shall be fulfilled only where there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical feature" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, make over the prior art."

The problem addressed by the application is to develop a method to direct differentiation of human ES cells towards a useful cell type and to generate the cell type in high yield to improve the chances of successful transplantation (see page 3 lines 25-34). The present application discloses a method of directing human ES cells to a neural progenitor cell, methods of directing a neural progenitor cell to a specific neural cell fate, particularly a midbrain fate and a method to enhance survival of transplanted dopaminergic neurons. The general concept underlying the application appears to reside in the generation or use of neural progenitor cells. However, the claims are not all limited to using the specific method of generating the neural progenitor cells, using FGF-2 and a BMP antagonist, disclosed in the specification. Other methods of directing human ES cells to differentiate into neural progenitor cells are known in the prior art as admitted by the Applicant (see page 3 lines 25-33), and shown in the following documents:

Reubinoff BE *et al.* Nat Biotechnol. (2001) 19(12):1134-40 "Neural progenitors from human embryonic stem cells".

WO 01/98463 (Monash University *et al.*) 27 December 2001.

WO 01/68815 (Monash University *et al.*) 20 September 2001.

Thus, the use or generation of neural progenitor cells from human ES cells cannot be considered a "special technical feature". Thus the claims have been broadly grouped into three separate inventions. The groups of inventions and their inventive concepts are described below:

INVENTION GROUP 1:

This group refers to methods of directing the fate of human ES cells towards a neural progenitor cell using the combination of an FGF and a BMP antagonist. Claims 1-23 and 33-37 (completely) and claims 31 and 32 (in part) form part of this group. However, there are broad claims within this group that are not limited to the technical features of this invention, namely the combination of FGF and a BMP antagonist, namely, claims 7 and 22 (and dependent claims 8-10, 19-21 and 23). These broad claims will only be searched with respect to the combination of FGF and a BMP antagonist being used to direct the human ES cells towards a neural progenitor cell.

**Supplemental Box**
(To be used when the space in any of Boxes I to VIII is not sufficient)

**Continuation of Box No: II**

INVENTION GROUP 2:

This group refers to methods of directing differentiation of neural progenitor cells to a midbrain fate. Claims 24-30 and 38-81 (completely) and claims 31, 32 and 83-92 (in part) form part of this group. It is admitted that not all the claims are directed to producing a midbrain fate. However, it is considered that the specification only provides support for the differentiation of neural progenitor cells to a midbrain fate. Therefore those claims that are not limited to producing a midbrain fate, namely claims 24-26, 28-31, 53, 64, 68 and 76-81, are not considered to comprise all the technical features of the invention. Consequently, these claims will only be searched with respect to directing towards a midbrain fate.

INVENTION GROUP 3:

This group refers to methods of enhancing survival of transplanted dopaminergic neurons by inducing expression of GDNF and BDNF. Claim 82 (and dependent claims 84-92) (completely) and claim 83 (and dependent claims 84-92) (in part) form part of this group. It is acknowledged that claim 83 comprises two separate options, one of these links the differentiated neural progenitor cells back to invention group 2, the other option is completely independent and does not require any specific method for obtaining the neural progenitor cells. Consequently, this second option does not share any "special technical features" with the other groups of inventions. Therefore these claims together with claim 82 constitute a third invention group.

Although the claims have been divided into three inventions there are certain broad claims within each invention that do not fit perfectly within one group. For example claim 22, which reads:

> "An isolated neural progenitor cells differentiated from hES cells and wherein the neural progenitors are not committed to a neural fate"

This claim is very broad and does not define the means by which the hES cells are caused to differentiate into a neural progenitor cell. Hence, this claims is considered not to define the technical features of the invention because the claim does not comprise the combination of FGF and a BMP antagonist to direct differentiation of the hES cells. Despite this, the claim has been placed in invention group 1. Hence, although this claim deals with differentiating hES cells to neural progenitor cells it also has some overlap with inventions 2 and 3. The same can be said for claim 64, which shows some overlap between inventions 2 and 3. However, the overlap is a consequence of overly broad claims and not from some shared "special technical feature". In fact as a consequence of the broad scope of these claims the claims that show overlap between the inventions are not novel in light of any one of the documents cited above.

| **INTERNATIONAL SEARCH REPORT**<br>Information on patent family members | International application No.<br>**PCT/AU03/00704** |
|---|---|

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Member (To put a line under the citations tab to the first point on the next row and press F8) | | | | | |
|---|---|---|---|---|---|---|---|
| WO | 200226941 | AU | 200193586 | CA | 2424062 | US | 2002164791 |
| WO | 2002081663 | JP | 2002291469 | | | | |
| WO | 200168815 | AU | 20006211 | AU | 200140361 | CA | 2403000 |
| | | EP | 1263932 | US | 2002068045 | US | 2002164308 |
| | | AU | 20001279 | AU | 20012920 | CA | 2406610 |
| | | EP | 1302536 | | | | |
| WO | 200027995 | AU | 200015015 | CA | 2349415 | EP | 1129176 |
| | | US | 2002160509 | | | | |
| WO | 200198463 | AU | 20008242 | AU | 200165704 | CA | 2411914 |
| | | EP | 1294855 | US | 2002022267 | AU | 20001327 |

END OF ANNEX

*(To add more lines press TAB at end of last row, remove paragraph marker to join up 'END OF ANNEX' box)*